PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 9/10, 15/63, C12P 21/06, A61K 38/51

A1

(11) International Publication Number: WO 96/12797

(43) International Publication Date: 2 May 1996 (02.05.96)

(21) International Application Number: PCT/US95/13691

(22) International Filing Date: 19 October 1995 (19.10.95)

(30) Priority Data:
08/326,774 19 October 1994 (19.10.94) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 21st floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).

(72) Inventors: MACLAREN, Duncan, C.; 21st floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US). MUDGETT, Mary, Beth; 21st floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US). CLARKE, Steven; 21st floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson and Bear, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PRODUCTION AND USE OF HUMAN AND PLANT METHYLTRANSFERASES

(57) Abstract

An isolated recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase is obtained by overexpression of cDNA coding for isozyme II in an *E. coli* strain, and a cDNA clone of the wheat enzyme and a purified enzyme from wheat are obtained. These enzymes are useful in treatment of medical conditions and diagnosis of disease associated with an increase in L-isoaspartyl/D-aspartyl residues of polypeptides in a tissue.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SI	Slovenia
SK	Slovakia
SN	Senegal
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

PRODUCTION AND USE OF HUMAN AND PLANT METHYLTRANSFERASES

BACKGROUND

Field of the Invention

The present invention relates to the production and use of methyltransferases. More specifically, the invention relates to the production, purification and use of recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase, an isolated polynucleotide coding for a plant L-isoaspartyl protein methyltransferase and a purified plant L-isoaspartyl protein methyltransferase.

Background of the Invention

Proteins undergo spontaneous thermodynamically driven changes over time that can result in decreased functionality (Harding, J.J. (1985) *Adv.Protein Chem.* 37:247-334). A common form of such damage is the nonenzymatic deamidation, isomerization, and racemiziation of asparaginyl and aspartyl residues (Clarke, et al. (1992) "Stability of Protein Pharmaceuticals, Part A: Chemical and Physical Pathways of Protein Degradation" pp1-29, Plenum Press, New York). In most cells, proteins containing L-isoaspartyl and D-aspartyl residues are recognized by the protein-L-isoaspartate (D-aspartate) *O*-methyltransferase (E.C. 2.1.1.77) which catalyzes their methyl esterification (McFadden, et al. (1982) *Proc.Natl.Acad.Sci. USA* 79:2460-2464, Murray, et al. (1984) *J.Biol.Chem.* 259:10722-10732, and Aswad (1984) *J.Biol.Chem.* 259:10714-10721). The enzymic methylation reaction represents the first step of a process that results in the conversion of these altered aspartyl residues to normal L-aspartyl residues (McFadden, et al. (1987) *Proc.Natl. Acad.Sci.USA* 84:2595-2599, Johnson, et al. (1987) *J.Biol.Chem* 262:5622-5629, Lowenson, et al. (1991) *J.Biol.Chem.* 266:19396-19406, Lowenson, et al. (1992) *J.Biol.Chem.* 267:5985-5995). This reaction prevents the accumulation of proteins containing isomerized and racemized aspartyl residues and may be an important component in limiting the detrimental effects of the aging process. In bacteria, it has been shown that the enzyme is crucial to stationary phase and heat-shock survival since deletion mutations decrease survival by up to 100-fold (Li, et al. (1992) *Proc.Natl.Acad.Sci. USA* 89:9885-9889)

L-isoaspartyl/D-aspartyl methyltransferases have been extensively studied in mammalian tissues. Two similar activities have been isolated from bovine brain (Aswad, et al. (1983) *J. Neurochem.* 40:1718-1728) and human erythrocytes (Gilbert, et al. (1988) *Biochemistry* 27:5227-5233, Ota, et al. (1988) *Biochem. Biophys. Res. Commun.* 151:1136-1143). These isozymes are monomeric polypeptides of about 25,000 Da and have similar catalytic properties, but differ by about 1 pH unit in isoelectric point (Ota, et al. (1988) *Biochem. Biophys. Res. Commun.* 151:1136-1143). A third activity has been noted, with an isoelectric point between isozymes I and II, but the corresponding isozyme has not been isolated or characterized (Ingrosso, et al. (1991) *Adv. Exper. Med. Biol.* 307:263-276). The complete amino acid sequences of isozyme I from bovine brain (Henzel, et al. (1989) *J. Biol. Chem.* 264:15905-15911) and human erythrocytes (Ingrosso, et al. (1989) *J. Biol. Chem.* 264:20131-21039) as well as most of that of isozyme II from human erythrocytes (Ingrosso, et al. (1991) *Biochem. Biophys. Res. Commun.* 175:351-358) have been determined. The sequence of the human isozyme I is 96% identical to the bovine enzyme. The amino acid sequences of the two human isozymes appear to be identical with the exception of two amino acids

at the C-terminus which may account for the difference in isoelectric point (Ingrosso, et al. (1991) *Biochem. Biophys. Res. Commun.* 175:351-358).

Southern blot analysis suggests that both isozymes are products of a single gene (Ingrosso, et al. (1991) *Biochem. Biophys. Res. Commun.* 175:351-358). Nucleotide sequences encoding enzymes similar to isozyme I in rat brain and mouse testis have recently been determined (Sato, et al. (1989) *Biochem. Biophys. Res. Commun.* 161:342-347, Romanik, et al. (1992) Gene, 118:217-222).

Two cDNA clones corresponding to the mRNAs for two isozymes of the human L-isoaspartyl/D-aspartyl protein carboxyl methyltransferase (EC 2.1.1.77) (MacLaren, et al.(1992) *Biochem. Biophys. Res. Commun.* 185, 277-283) have been sequenced. The DNA sequence of one of these (pRK1) encodes the amino acid sequence of the C-terminal half of the human erythrocyte isozyme I. The other cDNA clone (pDM2) includes the complete coding region of the more acidic isozyme II. With the exception of potential polymorphic sites at amino acid residues 119 and 205, the deduced amino acid sequences differ only at the C-terminus, where the -RWK sequence of isozyme I is replaced by a -RDEL sequence in isozyme II. The latter sequence is identical to a mammalian endoplasmic reticulum retention signal. The missing portion of the coding region for pRK1 is assumed to match that of pDM2. The presence of alternative splicing suggests the existence of a third isozyme having a -R C-terminus. Finally, evidence has been presented for the existence of three genomic polymorphisms in the human gene for this enzyme. Residue 22 can be either isoleucine (I) or leucine (L); residue 119 can either be isoleucine or valine (V); and residue 205 can either be lysine (K) or arginine (R) (Tsai and Clarke (1994) *Biochem. Biophys. Res. Commun.* 203:491-497). Thus, each isozyme described above can exist in at least eight forms ($I_{22}I_{119}K_{205}$, $I_{22}I_{119}R_{205}$, $I_{22}V_{119}K_{205}$, $I_{22}V_{119}R_{205}$, $L_{22}I_{119}K_{205}$, $L_{22}I_{119}R_{205}$, $L_{22}V_{119}K_{205}$, $L_{22}V_{119}R_{205}$).

However, in the above studies, the DNAs coding for the human methyltransferase were not efficiently expressed due to the lack of suitable recombinant expression systems. Thus, large amounts of homogenous enzyme have not been previously isolated. Methyltransferase has been purified from bovine brain where 3.7 mg was obtained in 5 steps from 0.58 kg of cerebral cortex (Aswad, et al. (1983) *J.Neurochem.* 40:1718-1726.). The human enzyme has been more difficult to purify, i.e., only about 0.1 mg of protein was obtained in 5 steps from 0.22 liters of blood (Gilbert, et al. (1988) *Biochemistry* 27:5227-5233.). Accordingly, efficient purification systems to produce large quantities of isoaspartyl methyltransferases on an industrial scale have heretofore not been available.

In plants, a protein L-isoaspartyl methyltransferase activity has been identified in both the monocots and the dicots as well as the green algae, *Chlamydomonas reinhardtii* (Mudgett, et al. (1993) *Biochemistry* 32:11100-11111). Although some enzymatic activity is present in most plant organs, the levels can vary considerably. Interestingly, the highest level of L-isoaspartyl methyltransferase activity is found in seeds (Mudgett, et al. (1993) *Biochemistry* 32:11100-11111). Moreover, the *in vitro* formation of carboxyl methylated proteins in the soluble fraction of seeds in the absence of exogenous peptide substrates suggests that methyl-accepting substrates exist for the methyltransferase *in vivo* (Trivedi, et al. (1982) *Eur. J. Biochem.* 128:349-354 and Mudgett, et al. (1993) *Biochemistry* 32:11100-11111).

The level of isoaspartate in proteins has been used as an indication of the level of damage to the proteins. Methods for determining the isoaspartyl content of proteins using isoaspartyl methyl transferase enzymes are disclosed in U.S. Patent No. 5,273,886 to Aswad, the disclosure of which is hereby incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows construction of the human L-isoaspartyl/D-aspartyl methyltransferase expression plasmid, pDM2x of the present invention. A 107bp *KpnI-NarI* fragment is removed from plasmid pDM2 and replaced with a synthetic polylinker containing ribosome binding and initiator sites.

FIGURE 2 shows the novel polylinker fragment used in the present invention. The polylinker contains multiple cloning sites (*KpnI*, *KhoI*, *XbaI*, *BamHI*, *NheI*, *NcoI*) and a strong prokaryotic ribosomal binding site (Shine, et al. (1974) *Proc. Natl. Acad. Sci. USA* 71:1342-1346).

FIGURE 3 shows the nucleotide and deduced amino acid sequence of the human L-isoaspartyl/D-aspartyl methyltransferase expression plasmid, pDM2x, of the present invention (SEQ ID NO:8).

FIGURE 4 shows the effect of isopropyl β-D-thiogalactopyranoside (IPTG) concentration on the level of human L-isoaspartyl/D-aspartyl methyltransferase production in *E. coli*.

FIGURE 5 shows E. coli growth percent of total soluble protein obtained as human methyltransferase, and yield of human methyltransferase in the pDM2x expression system of the present invention as a function of time after IPTG induction.

FIGURE 6 shows optimization of the protamine sulfate precipitation step according to the present invention.

FIGURE 7 shows optimization of ammonium sulfate precipitation fractionation of human L-isoaspartyl/D-aspartyl methyltransferase from the protamine sulfate clarified supernatant according to the present invention.

FIGURE 8 shows the anion exchange column purification step of the human L-isoaspartyl/D-aspartyl methyltransferase according to the present invention.

FIGURE 9 shows the pH dependence for concentrating purified human L-isoaspartyl/D-aspartyl methyltransferase according to the present invention.

FIGURE 10 shows electrospray mass spectral analysis of purified recombinant human methyltransferase of the present invention. (A) A portion of the spectrum of material purified using dithiothreitol. (B) A portion of the spectrum of material purified when 15mM β-mercaptoethanol was substituted for 0.1 μM ditiothreitol in buffer A.

FIGURE 11 shows the ultraviolet absorbance spectrum of the purified human L-isoaspartyl/D-aspartyl methyltransferase of the present invention.

FIGURE 12 shows the purification of wheat germ L-isoaspartyl methyltransferase according to the present invention. (A) DEAE-52 anion exchange cellulose treatment. (B) Reverse ammonium sulfate gradient solubilization treatment. (C) Sephacryl S-200 gel filtration.

FIGURE 13 shows the Polypeptide analysis of the purification of L-isoaspartyl methyltransferase from wheat germ according to the present invention.

FIGURE 14 shows the DNA sequencing strategy of the wheat germ methyltransferase cDNA insert employed in the present invention.

FIGURE 15 shows an alignment of the sequenced peptide fragments of L-isoaspartyl methyltransferase from wheat germ and its predicted amino acid sequence from pMBM1.

SUMMARY OF THE INVENTION

One particular objective of the present invention is to provide an isolated recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase by expression of the cDNA encoding the enzyme. Surprisingly, this expression system permits an extremely efficient large-scale production of a pure recombinant enzyme. The structure of the recombinant enzyme is different from that of the purified enzyme from human erythrocytes only at the N-terminal alanine residue where the recombinant enzyme is not modified by the post-translational modification of acetylation. Thus, this enzyme is suitable for use in human studies without the potential problem of antigenicity.

Other objectives of the present invention include the identification of cDNA encoding wheat L-isoaspartyl protein methyltransferase, and the provision of a purified plant L-isoaspartyl protein methyltransferase from wheat. High levels of methyltransferase are found in wheat, especially in seeds.

Still other objectives of the present invention are to provide a pharmaceutical preparation containing the enzyme to treat disorders resulting from protein degradation, and to provide an analytical tool for quality control of protein and peptide pharmaceuticals and for diagnosis of disease states associated with protein degradation. Other objectives of the present invention will become apparent to one having ordinary skill in the art upon reference to the ensuing detailed description of the invention.

Namely, one aspect of the present invention is an isolated recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase obtained by expression of a polynucleotide having a sequence selected from the group consisting of SEQ ID NO:1 (isozyme I) and SEQ ID NO:2 (isozyme II), each including a total of 17 nucleotide sequences coding for eight amino acid sequences of isozyme I or II, i.e., $I_{22}I_{119}K_{205}$, $I_{22}I_{119}R_{205}$, $I_{22}V_{119}K_{205}$, $I_{22}V_{119}R_{205}$, $L_{22}I_{119}K_{205}$, $L_{22}I_{119}R_{205}$, $L_{22}V_{119}K_{205}$, and $L_{22}V_{119}R_{205}$ as described earlier.

Another aspect of the present invention is an isolated recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase having an amino acid sequence selected from the group consisting of SEQ NO:3 (isozyme I) and SEQ ID NO:4 (isozyme II), each including eight amino acid sequences as above.

Another aspect of the present invention is an isolated polynucleotide having the coding sequence of the sequence indicated as SEQ ID NO:5, which codes for a plant L-isoaspartyl protein methyltransferase. The 690 base pairs of this sequence beginning with the ATG codon at base numbers 117-199 and ending with the AGC codon prior to the TGA codon at positions 807-809 represent the coding sequence of this polynucleotide.

Another aspect of the present invention is an isolated recombinant plant L-isoaspartyl protein methyltransferase obtained by expression of a polynucleotide having the sequence indicated as SEQ ID NO:5.

Another aspect of the present invention is a purified plant L-isoaspartyl protein methyltransferase from wheat germ having the amino acid sequence indicated as SEQ ID NO: 6 or 7.

Another aspect of the present invention is a method of recombinantly producing human L-isoaspartyl/D-aspartyl protein methyltransferase, comprising:

modifying a plasmid such as plasmid pDM2 (Genebank accession # S37495) that contains the full coding region of human L-isoaspartyl/D-aspartyl protein methyltransferase, using oligonucleotides, to provide multiple cloning sites, an efficient ribosome binding site, and a strong translational initiator region, said initiator region being designed to function in bacterial and/or eukaryotic expression system;

transfecting the constructed vector into a host that contains an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible T7 polymerase gene; and

inducing overexpression of the methyltransferase with IPTG, whereby the methyltransferase is produced. Also, any system producing T7 polymerase gene can express the methyltransferase.

This expression method is advantageously applicable to any variants of the methyltransferase. The enzyme is preferably obtained from an overexpressed human cDNA in *E. coli* such as BL21(DE3) grown in LB Broth. Further efficient and economical expression is achieved using a richer media, e.g., terrific broth (Sambrook, et al. (1989) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory), which results in a higher final cell density and a longer exponential growth and methyltransferase production phase. In the present invention, the final result can be a preparation where the human methyltransferase makes up about 10-30% of the total bacterial soluble protein.

Another aspect of the present invention is a method of purifying recombinantly produced human L-isoaspartyl/D-aspartyl protein methyltransferase present in a lysed bacterial extract in which the methyltransferase expression has been performed, comprising:

adding a nucleotide precipitant such as protamine sulfate or polyethyleneimine to the extract to remove DNA present in the extract subsequent to removing the cellular debris;

precipitating the methyltransferase with ammonium sulfate;

removing the ammonium sulfate by dialysis; and

purifying the methyltransferase from the dialysate using anion-exchange chromatography under novel conditions.

Based on this novel purification, it is possible to obtain about 50 mg of enzyme from 4 liters of bacterial culture. The success of a single column in purifying this enzyme is attributed to finding conditions where the enzyme can be weakly bound to the column and eluted isocratically in starting buffer without the application of a salt gradient. It is found that a DEAE-cellulose column not fully equilibrated with a phosphate/EDTA buffer gave the best fractionation.

Another aspect of the present invention is a method of purifying plant L-isoaspartyl protein methyltransferase from wheat, comprising:

obtaining a crude cytosol from raw wheat germ;

fractionating the crude cytosol by DEAE-cellulose chromatography;

adding ammonium sulfate to the pooled active fractions in the presence of a protein carrier;

fractionating the resulting material by reverse ammonium sulfate gradient solubilization; and

purifying the pooled active fractions by gel filtration chromatography, whereby the methyltransferase is purified as a monomeric 28,000 Da species.

Another aspect of the present invention is a method of treatment for a medical conditions associated with an increase in L-isoaspartyl/D-aspartyl residues of polypeptides in a tissue, comprising administering to the tissue an amount of methyltransferase, with or without S-adenosylmethionine, sufficient to convert said L-isoaspartyl/D-aspartyl residues to L-aspartyl residues in the tissue.

According to another aspect of this preferred embodiment, there is provided a method of diagnosis of disease states where L-isoaspartyl/D-aspartyl residues are accumulated, comprising measuring the content of L-isoaspartyl/D-aspartyl residues accumulated in a disease associated protein, by using methyltransferase as a probe.

Another aspect of the invention is a method of determination of degradation of pharmaceutical polypeptides, comprising measuring the content of L-isoaspartyl and D-aspartyl residues in the polypeptides, by using methyltransferase as a probe.

Still another embodiment of the invention is a pharmaceutical preparation for treatment of a medical condition associated with an increase in L-isoaspartyl/D-aspartyl residues of polypeptides in a tissue, comprising human L-isoaspartyl/D-aspartyl protein methyltransferase, preferably with its substrate S-adenosylmethionine, and pharmaceutically acceptable carriers.

Since large amounts of the enzyme can be obtained by the present invention, the commercial utility of this enzyme becomes applicable. The methyltransferase can be used as a sensitive analytical probe of L-isoaspartyl and D-aspartyl residues in quality control of protein and peptide pharmaceuticals. The uses in medical diagnostics for assaying for altered proteins and peptides in biological fluids such as the β-amyloid product (Roher, A.E., et al. (1993) *J.Biol.Chem.* 268:3072-3083) is possible. Because the endogenous human methyltransferase is limited to the cytosol (Clarke, S. (1985) *Annu Rev Biochem* 54:479-506), damaged proteins in the extracellular environment cannot undergo repair catalyzed by this enzyme. Thus, injectable and topical therapeutic preparations using the methyltransferase and its substrate S-adenosylmethionine are useful. In particular, the usefulness of this enzyme in repairing damaged proteins in skin such as collagen and elastin whose degradation may contribute to the aging of this tissue is advantageous.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Overexpression of the Human L-Isoaspartyl/D-Aspartyl Methyltransferase in *E. coli*

The plasmid pBluescript SK(-) (pDM2: Genebank accession # S37495) contains the entire coding region (SEQ ID NO:9) for the more acidic isozyme II of the human L-isoaspartyl/D-aspartyl methyltransferase (SEQ ID NO:9) (MacLaren, et al. (1992) *Biochem. Biophys.Res.Commun.* 185:277-283.). This plasmid is obtained from a cDNA library derived from HUMAN brain tissue (Stratagene, La Jolla, CA) using a mouse cDNA as a probe. As an expression vector, a plasmid containing cDNA encoding isozyme I (SEQ ID NO:1) or isozyme II (SEQ ID NO:2) having sequences other than pDM2 (SEQ ID NO:9) can be used to produce isozyme I having the sequence indicated as SEQ ID NO:3 or isozyme II having the sequence indicated as SEQ ID NO:4 so that each isozyme can be produced in at least eight forms ($I_{22}I_{119}K_{205}$, $I_{22}I_{119}R_{205}$, $I_{22}V_{119}K_{205}$, $I_{22}V_{119}R_{205}$, $L_{22}I_{119}K_{205}$, $L_{22}I_{119}R_{205}$, $L_{22}V_{119}K_{205}$,

$L_{22}V_{119}R_{205}$) as described earlier. Alternatively, isozyme III with a -R C-terminus (226 residue long) can be produced in the same manner. Such plasmids include pBK phage vector (Strategene) and the pTrc99A expression plasmid (Pharmacia, Piscatway, NJ). *E. coli* is preferred for expression of human methyltransferase because characterization of isozyme II purified from human erythrocytes has shown that it is not post-translationally modified (Ingrosso, et al. (1989) *J.Biol.Chem.* 264:20131-20139.). Thus, the *E. coli* expression system can produce the recombinant methyltransferase nearly identical to the human enzyme. The pDM2 plasmid already contains a T7 promoter site in the proper position and orientation for transcription of the insert cDNA but does not contain a ribosomal binding site (MacLaren, et al. (1992) *Biochem. Biophpys.Res.Commun.* 185:277-283.). Thus, the pDM2 plasmid is modified to give the overexpression vector, pDM2x, by replacing the region between the T7-promoter site and the start codon of the enzyme with a synthetic fragment containing a strong ribosomal binding site (Hine, et al. (1974) *Proc.Natl.Acad.Sci. USA* 71:1342-1346.) (Fig. 1). In Fig. 1, the *KpnI-NarI* fragment from pDM2 is replaced with a synthetic linker containing multiple cloning sites, a eukaryotic initiator site, and a strong ribosomal binding site (Fig. 2). The fragment shown in Fig. 2 was generated by polymerase chain reaction (PCR) using oligonucleotide primers containing the *KpnI* and *NarI* restriction sites for proper insertion. The regions 5' from the *KpnI* restriction site, including the T7-promoter site, were originally part of the pBluescript SK(-) plasmid. The sequences 5' of the *NcoI* start are not present in the original methyltransferase cDNA insert. pDM2 is a pBluescript SK(-) (Stratagene) plasmid containing the human methyltransferase isozyme II cDNA inserted into its *Eco*RI sites. Fragment sizes given exclude the *KpnI* and *NarI* 4 and 2 base-pairs overhangs, respectively. This replacement fragment has been engineered to also possess multiple cloning sites for the insertion the methyltransferase cDNA (Fig. 3) into different expression systems and organisms. In Fig. 3, the *Eco*RI site at the 3'-end of the sequence shows 7 bases that are part of the pBluescript SK(-) cloning plasmid. The regions 5' from the *KpnI* restriction site, including the T7-promoter site, are part of the pBluescript SK(-) plasmid also. The fragment has the sequence SEQ ID NO:8. The nucleotide and translated amino acid sequence of the pDM2x expression plasmid from the T7-promoter site to the 3' *Eco*RI linker on the tail of the human cDNA insert is shown in Fig. 3. The structure of the modified region of pDM2x and the overall plasmid structure have been confirmed by DNA sequence analysis and restriction endonuclease digest analysis, respectively. Any synthetic polylinker containing ribosome binding and initiator sites capable of being inserted into a methyltransferase-encoding vector are within the scope of the present invention. The T7 RNA polymerase-driven expression plasmid is then transfected into *E. coli* strain BL21(DE3) (Studier, et al. (1986) *J. Mol. Biol.* 189:113-130) for expression of the human L-isoaspartyl/D-aspartyl methyltransferase. This strain of bacteria contains a phage T7 RNA polymerase gene in the chromosome under the control of the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *lac*UV5 promoter. Other bacterial host strains containing an IPTG-inducible T7 polymerase gene are also contemplated. For example, it is possible to use a dual plasmid system where the T7 polymerase is encoded behind a heat-inducible promoter on plasmid pGP1-2. This plasmid can be transformed into a variety of bacterial strains.

Methyltransferase Purification

The initial batch purification steps used here to enrich the methyltransferase fraction in the lysed bacterial extract is a modification of the procedure used by Fu, *et al.* (Fu, et al. (1991) *J.Biol.Chem.* 266:14562-14572.).

After lysis of the overexpressing bacteria by sonication, the cellular debris is removed by centrifugation. The nucleic acids remaining in the supernatant are removed by addition of a precipitant, protamine sulfate or polyethyleneimine, and centrifugation. The amount of protamine sulfate required is optimized, and it is found that addition of 0.1 volumes of a 4% solution of protamine sulfate gives a good purification of methyltransferase in essentially quantitative yield. The methyltransferase then is concentrated and purified further by precipitation with ammonium sulfate. Again, conditions for this step in preliminary experiments are optimized, and it is found that although the best purification occurs between 50 to 55% saturation, the best compromise between yield and purification occurs at 60% saturation and these conditions are used in the present large-scale purification. The pelleted protein is then resuspended in a small volume of buffer A and dialyzed against buffer A to remove the ammonium sulfate.

Then a rapid, one-step column procedure for purifying the methyltransferase from the dialysate by using DEAE-cellulose anion exchange chromatography is performed. It is found that application of traditional methodologies where the enzyme was bound to a column in a noninteracting cationic buffer resulted in incomplete purification. However, the use of an interacting anionic buffer under nonequilibrium conditions was found to result in the isocratic elution of homogenous enzyme. If the chromatography is performed where the column is fully equilibrated before loading, then the methyltransferase is only slightly retarded and elutes close to the void volume along with small amounts of contaminating polypeptides (data not shown).

Identification of L-Isoaspartyl Methyltransferase in Plants

Peptide-dependent L-isoaspartyl methyltransferase is found in the vegetative cells of the green alga *C. reinhardtii*, demonstrating its presence in at least one species in the Kingdom Protista. In the Kingdom Plantae, methyltransferase activity is detected in both classes of the angiosperms, the monocots and the dicots. The level of activity in different tissues varies considerably. Of the species assayed, the highest specific activity of the methyltransferase is found in wheat embryos (germ). In contrast, almost no detectable L-isoaspartyl peptide-specific methyltransferase activity is found in the leaves of lettuce or the fruits of tomato. The specific activity of the enzyme in wheat germ (14.0 pmol/min/mg) surpasses the levels found in *E. coli* (1-2.5 pmol/min/mg; Fu, et al. 1991) and human erythrocytes (1.9 - 9.4 pmol/min/mg; Ota, et al., 1988; Gilbert, et al. 1988). Thus, wheat germ, a cheap and abundant byproduct of wheat flour production, is an excellent source of material for enzyme purification."

L-Isoaspartyl Methyltransferase in Wheat Seeds and Seedlings

Peptide-dependent L-isoaspartyl methyltransferase activity is highest in mature wheat seeds and the activity is significantly reduced following imbibition and germination. Northern analysis shows that methyltransferase mRNA is expressed as a single 1200 nucleotide species only in seeds, and not in whole seedlings, leaves, or roots. The levels of the enzyme vary depending on stages of caryopsis development, and the highest level of methyltransferase mRNA is detected in stage IV seeds whose embryos have reached maximal size while no methyltransferase mRNA is detectable at stage II. Northern analysis shows that 10 h treatments of water deficiency, exposure to 50 μM (+)-*cis,trans*-abscisic acid) (ABA), and exposure to salt stress (0.25 M NaCl) dramatically induce the expression of methyltransferase mRNA in wheat seedlings. In contrast, methyltransferase gene expression is not induced in seedlings exposed to low (4°C) or high (37°C) temperature stress. These results indicate that the induction of L-

isoaspartyl methyltransferase mRNA expression and enzymatic activity occurs not only in seed development and germination, but can also be upregulated in seedlings during periods of water deficit and salt stress. In particular, when the seedlings are treated with both ABA and NaCl, methyltransferase gene expression is increased approximately two-fold over the effect of either agent alone. The additive effect of a combined ABA-NaCl treatment suggests that the methyltransferase gene may be a salt-responsive gene in addition to an ABA-responsive gene. The hormonal and environmental stress necessary for inducing expression of methyltransferase is preferably an ABA concentration of 10-100 μM, a salt concentration of 0.1-1 M, or a dehydration time of 5-24 hours (Although dehydration may be larger for other plants up to 7 days).

Purification of L-Isoaspartyl Methyltransferase from Wheat Germ

Because of its high methyltransferase activity, the wheat system was chosen. The present purification strategy is based on the partial purification of the protein carboxyl methyltransferase reported by Trivedi, et al. (Trivedi, et al. (1982) *Eur. J. Biochem.* 128, 349-354). Methyltransferase is purified from a cytosolic fraction of raw wheat germ. This material is first fractionated by DEAE-cellulose chromatography at pH 7-10, preferably at 9.3 (Fig. 12A). Active fractions are then saturated to 60-100% , preferably to 80%, with ammonium sulfate in the presence of a protein carrier such as Celite 545, poured into a column, and fractionated by reverse ammonium sulfate gradient solubilization at room temperature (Fig. 12B). Active fractions containing approximately 20-50%, preferably 26-31% saturated ammonium sulfate, can be further purified preferably by using Sephacryl S-200 gel filtration chromatography, although other gel filtration materials are also contemplated. Surprisingly, the L-isoaspartyl methyltransferase elutes in a highly purified state in a fraction nearly corresponding to the total volume of the column. This step is unique in that the methyltransferase is not fractionated on the basis of its size. Rather it is suggested that the methyltransferase associates with the Sephacryl S-200 resin through hydrophobic interactions due to a solvent effect created by the relatively high concentration of ammonium sulfate in the fractions (Belew, et al. (1978) *J. Chromatogr.* 147, 205-212). In the absence of ammonium sulfate, the methyltransferase elutes from the Sephacryl S-200 column in a position consistent with a monomeric molecular weight, along with numerous contaminating polypeptides. Thus, the successful isolation of a highly purified enzyme preparation from the gel filtration column is attributed to this unusual absorption phenomenon.

This polypeptide corresponds to the L-isoaspartyl methyltransferase as assessed by renaturing individual gel slices in the presence of Triton X-100 as described by Clarke (Clarke (1981) Biochim. Biophys. Acta 670, 195-202). The purity of this preparation can be as high as 80-100%, estimated from densitometry of the Coomassie-stained gel (Fig. 13).

DNA Sequence of the Gene Encoding L-Isoaspartyl/D-aspartyl Methyltransferase from Wheat

The DNA sequence of the 952-bp cDNA insert in the plasmid pMBM1 is determined using the sequencing strategy shown in Fig. 14. The DNA sequence of the methyltransferase cDNA and its deduced amino acid sequence are indicated as SEQ ID NOs:5 and 6, respectively. The calculated molecular weight of the 230 amino acid polypeptide deduced for the 690-bp open reading frame is 24,710. In contrast, purified methyltransferase migrated as a 28,000 Da polypeptide as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Comparison of Sequenced Peptide Fragments of L-Isoaspartyl methyltransferase from Wheat Germ and Its Predicted Amino Acid Sequence from pMBM1

Interestingly, discrepancies at 12 sites between the predicted amino acid sequence of the wheat cDNA and the sequence of the peptide fragments of the wheat germ L-isoaspartyl methyltransferase are found (Fig. 15). In six of these positions, the experimentally determined amino acid sequence data clearly show the presence of an amino acid not encoded by the cDNA. At the other six positions, residues in addition to the encoded residue are identified by Edman degradation. These results are consistent with the hexaploid nature of this species of wheat, where the three diploid genomes (AABBDD) can contain alleles with variant sequences, leading to the production of variant gene products (Peumans, et al., (1982) *Planta* 154, 562-567 and Wright, et al. (1989) *J. Mol. Evol.* 28, 327-336). Most of the amino acid changes are located outside of the three highly conserved regions shared among methyltransferases. It is interesting to speculate that these amino acid differences can result in enzymes having slightly different methyl acceptor specificities, which would give the cell the ability to recognize and potentially repair a wider range of damaged proteins. Polymorphisms in the human methyltransferase gene have also been identified (Ingrosso, et al. (1989) *J. Biol. Chem.* 264, 20131-20139 and MacLaren, et al. (1992) *Biochem. Biophys. Res. Commun.* 185, 277-283).

Production of Isolated Recombinant Wheat Germ L-isoaspartyl Protein Methyltransferase

The methyltransferase cDNA insert is inserted into well-known prokaryotic expression vectors as described for the human enzyme and used to transform competent *E. coli*, followed by induction of the T7 polymerase gene with IPTG. The expression of the plant enzyme can be done in exactly the same manner as the human enzyme. The bacterial expression system is designed to express cDNA sequences regardless of their phylogenetic origin. The expressed recombinant protein is purified as described above.

Applications for L-isoaspartyl Methyltransferase

Methyltransferase catalyzes the S-adenosylmethionine-dependent methylation of atypical L-isoaspartyl and D-aspartyl residues in peptides and proteins. This reaction can not only be used as an analytical tool to detect the presence of these altered residues in aged and stressed proteins, but can also initiate a non-enzymatic pathway that can result in the conversion of these residues to normal L-aspartyl residues.

The methyltransferase enzymes of the present invention can be used in connection with the determination of L-isoaspartate and D-aspartyl residues in peptides as disclosed in U.S. Patent No. 5,273,886 to Aswad, incorporated herein by the previous reference thereto above. Briefly, this method involves breaking the polypeptide into fragments using a proteolytic enzyme and then quantitatively methylating the isoaspartyl residues in the fragments using a methyltransferase enzyme. The total amount of methyl groups incorporated into the fragments is an indication of the amount of isoaspartyl residues in the polypeptide. The amount of isoaspartyl residues in the polypeptide can be used as an indication of the amount of damage to proteins, such as those used in therapeutic applications.

Interestingly, the structure of the recombinant enzyme is different from that of the purified enzyme from human erythrocytes at the N-terminal alanine residue, and, as determined by electrospray mass spectroscopy, the

recombinant enzyme does not contain covalent post-translational modifications. Thus this enzyme is suitable for use in human studies without the potential problem of antigenicity. Because the endogenous human methyltransferase is limited to the cytosol (Clarke, S. (1985) *Annu Rev Biochem* 54:479-506), damaged proteins in the extracellular environment cannot undergo repair catalyzed by this enzyme. Thus, injectable and topical therapeutic preparations using the methyltransferase and its substrate S-adenosylmethionine are useful. Since the recombinant human enzyme has no potential problem of antigenicity, it may be injected directly into the brain, eye, blood stream and so forth. In addition, purified plant enzyme can be used in skin-care products as a topical preparation since it also recognizes damaged isoaspartyl residues in peptides and proteins.

By administering to a tissue an amount of methyltransferase, preferably in conjunction with its substrate S-adenosylmethionine, sufficient to convert said L-isoaspartyl/D-aspartyl residues to L-aspartyl residues in the tissue, treatment for a medical condition associated with an increase in L-isoaspartyl/D-aspartyl residues of polypeptides in a tissue can be performed. Such medical conditions include those resulting from crosslinking of matrix proteins and degradation of flexibility of skin tissues such as cataracts, Alzheimer's disease and the like. For this purpose, either the human or plant enzymes can be used, and the dosage of the enzyme is such that the concentration of the enzyme in the preparation is in the range of 0.4-40 μM. The enzyme can be formulated simply in the form of an ointment with S-adenosylmethionine and a pharmaceutically acceptable carrier. A typical ointment can contain the enzyme in an amount of 0.001-10% by weight and S-adenosylmethionine in an amount of 0.00004-0.4% by weight.

Other medical conditions are formation of plaque in brain tissues and degradation of cellular function in brain tissues, and, for these purposes, human enzyme is preferably used in an amount such that the concentration of the enzyme in the extracellular space is in the range of 0.4-40 μM. For administration to the brain, the enzyme can be provided as an injectable solution typically containing the enzyme in an amount of 0.001-10% by weight and S-adenosylmethionine in an amount of 0.00004-0.4% by weight in a pharmaceutically acceptable carrier. Preliminary evidence suggests that L-isoaspartyl and D-aspartyl residues can accumulate in the amyloid protein of Alzheimer's disease. Since a fraction of β-amyloid protein is found in the cerebrospinal fluid (CSF), it may also be possible to treat Alzheimer's disease by injecting the enzyme into the CSF.

Another medical condition is degradation of flexibility in a vascular system, and, for this purpose, human enzyme is preferably used in an amount such that the concentration of the enzyme in an erythrocyte, endothelial tissue, coronary artery tissue, immune cells, receptors of all cells or lungs is in the range of 0.4-40 μM. For the vascular system, the enzyme can be provided as an injectable intravenous solution typically containing the enzyme in an amount of 0.001-10% by weight and S-adenosylmethionine in an amount of 0.00004-0.4% by weight in a pharmaceutically acceptable carrier. The solution can be administered by means of a catheter or direct injection.

Other medical conditions are infertility related to eggs and/or sperm and formation of fibrosis in tissues, and, for these purposes, human enzyme is preferably used in an amount such that the concentration of the enzyme in egg or sperm cells is in the range of 0.4-40 μM. For the vascular system, the enzyme can be provided as an injectable solution typically containing the enzyme in an amount of 0.001-10% by weight and S-adenosylmethionine in an amount of 0.00004-0.4% by weight with a pharmaceutically acceptable carrier.

Since L-isoaspartyl and D-aspartyl residues are accurately recognized by methyltransferase, it is possible to determine the presence of these damaged residues in pharmaceutical polypeptides so that the purity and shelf-life of such protein products can be verified. These assays are performed by incubating the pharmaceutical preparation with S-adenosyl [^{14}C-*methyl*] methionine in the presence of the purified methyltransferase and determining the radioactivity transferred to the pharmaceutical. This is done by incubating the reaction products with an alkaline solution to release bound methyl esters as radioactive methanol, which is then collected in scintillation fluid as described (Lowenson, et al. (1991) *J. Biol. Chem.* 266:19396-19406).

Further, diagnosis of disease states in which L-isoaspartyl and D-aspartyl residues accumulate may be performed by measuring the content of L-isoaspartyl and D-aspartyl residues accumulated in a disease associated protein, by using methyltransferase as a probe. Since a fraction of β-amyloid protein is found in the cerebrospinal fluid (CSF), it is possible to develop a diagnostic test for Alzheimer's disease by measuring the content of L-isoaspartyl and D-aspartyl residues in samples of CSF. It has not hitherto been possible to obtain an accurate diagnosis of this disease which incapacitates millions of Americans. The diagnostic test can be accomplished using the same assay described above for protein pharmaceutical quality control.

EXPERIMENT 1: NUCLEOTIDE SEQUENCE OF HUMAN METHYLTRANSFERASE CODING REGION

cDNA Library Synthesis and Clone Screening

A cDNA library constructed from the temporal cortex of the brain of a 2-year-old female human was purchased from Stratagene (#935205). The cDNA was synthesized from oligo-dT isolated mRNA, and packaged into the *Eco*RI sites of the lambda ZAP bacteriophage vector (Stratagene). The library was propagated in *E. coli* BB4 and 22 plates containing 5 x 10^5 plaques each (1.1 x 10^7 plaques total) were screened using a radiolabeled 769 bp *HaeIII* fragment from the coding region of a 1580 bp murine methyltransferase cDNA (Romanik, et al. (1992) Gene, 118:217-222). The fragment was labelled with [α-^{32}P]-dCTP to a specific activity of 10^9 cpm/μg with the PRIME-IT random priming kit (Stratagene). Standard plaque lift and Southern blot procedures (Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory) produced three positive signals. The clones for these plaques were isolated by subsequent screenings. The clones were repackaged into plasmids in XL1-Blue cells via *in vivo* excision according to the λZAP protocol. Successful excision was denoted by ampicillin resistance. The cells containing the insert-carrying plasmids of interest were grown in LB/Ampicillin medium, and their plasmids isolated and purified using Qiagen plasmid isolation columns.

Nucleotide Sequence Determination and Analysis

The nucleotide sequences of the clones were determined on both strands by the dideoxy chain-terminating method (Sanger, et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467) using the Sequenase 2.0 kit (USB). M13 and T7 universal primers, and synthesized 22mer primers. The sequence data were analyzed with DNAStar programs on a Macintosh computer.

Three clones out of 1.1 x 10^7 plaques gave a positive signal and were isolated. The sequences of two of the clones (pDM2 (SEQ ID NO:9) and pRK1 (SEQ ID NO:10)) were determined from both strands. The plasmid pDM2 is available from Genebank under accession # S37495. In SEQ ID NOs:3, 4 and 9, the encoded initiator

methionine is numbered 0 and the next amino acid, alanine, is numbered one. This is done to match the numbering scheme of the final protein due to the excision of the initiator methionine. Numbering for both clones begins at these positions. Clone pRK1 begins at position 358. Numbers to the left of the divisor represent pDM2. Those to the right of the divisor are for pRK1. The nucleotide and encoded amino acid sequences of pDM2 is shown under (a) and continues to (b). (b) represents the 47 base insert found in clone pRK1.

EXPERIMENT 2: HUMAN METHYLTRANSFERASE EXPRESSION IN *E. COLI*

Construction of expression vector pDM2x

The pDM2 plasmid was modified to give the overexpression vector, pDM2x, by replacing the region between the T7-promoter site and the start codon of the enzyme with a synthetic fragment containing a strong ribosomal binding site, as previously described (Figs. 1-3).

Bacterial Growth

E. coli strain DH5α (Gibco-BRL, Gaithersburg, MD) was used for cloning and propagation of plasmid constructs. Transformation of *E. coli* was accomplished by the one-step method described by Chung, *et al. (Proc.Natl.Acad.Sci. USA (1989)* 86:2172-519). For protein expression, *E. coli* strain BL21(DE3) (Studier, et al. *J.Mol.Biol.*(1986) 189:113-130) was transformed with the pDM2x expression plasmid. BL21(DE3) bacteria containing the pDM2x plasmid were grown at 37°C in Luria-Bertrani (LB) broth (Sambrook, et al. "Molecular cloning: a laboratory manual," (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) medium containing 100 μg/ml ampicillin.

Protein Concentration Determination

Protein concentrations of crude extracts were determined by the trichloroacetic acid-Lowry method (Chang, Y.C. (1992) *Anal.Biochem.* 205:22-26) with bovine serum albumin as a standard. Protein concentrations of column fractions were determined by measuring the optical density at 280 nm and equating an absorbance of 1 to a concentration of 1.0 mg/ml for a mixture of proteins (Sambrook, et al.(1989) "Molecular cloning: a laboratory manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) or 1.12 mg/ml for homogenous methyltransferase (Mach, et al. *Anal.Biochem.* (1992) 200:74-80).

Methyltransferase Assay

The concentration of active methyltransferase was determined by measuring base-labile methyl ester formation on the methyl-acceptor ovalbumin using a vapor diffusion assay (Gilbert, et al. *Biochemistry (1988)* 27:5227-5233). Final concentrations in a 50 μL reaction mixture were 10 μM S-adenosyl-L-[*methyl*-^{14}C]methionine (53 mCi/mmole, 100 cpm/pmol, ICN Biomedicals, Irvine, CA), 40 mg/ml chicken ovalbumin (fraction V, Sigma, St. Louis, MO), and 0.2 M sodium citrate, pH 6.0. Incubations were done at 37°C for 30 min and quenched by the addition of an equal volume (50 μL) of 0.2 N NaOH, 1.0% (w/v) SDS. This mixture was spotted on a 1 cm x 9 cm piece of thick filter paper (No. 165-090, Bio-Rad, Richmond, CA) prefolded in an accordion pleat and placed in the neck of a 20 ml plastic scintillation vial containing 6 ml of Safety-Solve counting fluor (No. 111177, Research Products International). The vials were capped and, after 2 hours of incubation at 23°C, the filter paper inserts were removed and the vials recapped. Radioactivity was measured over a wide ^{14}C channel. Once purified to

homogeneity, as determined by SDS-PAGE, the specific activity of methyltransferase was measured (10,000 pmoles/min/mg at 37°C). This value was used to determine methyltransferase mass from enzyme activity measurements.

Methyltransferase Expression

Ten one liter cultures of *E. coli* strain BL21(DE3) containing expression plasmid pDM2x were grown at 37°C in LB broth containing 100 μg/ml ampicillin with shaking at 250 rpm to an optical density of 0.5 at 600 nm. Expression of the enzyme was then induced by adding IPTG to 50 μM. Cell growth was allowed to continue for 4 more hours until growth reached saturation at an optical density at 600 nm of 1.6. The cells were harvested by centrifugation at 5,000 g for 15 min at 4°C, yielding a 14 gram wet weight pellet. All operations from here until the column fractionation were done at 4°C.

To determine the optimal concentration of IPTG for expression, log-phase expression cultures were induced with a wide concentration range of IPTG and assayed for methyltransferase concentration. Fig. 4 shows that only very low amounts (0.03 mM) of IPTG were required to induce expression of the methyltransferase. In Fig. 4, a 1 liter solution of LB broth at 37°C containing ampicillin at 100 μg/ml was inoculated with BL21(DE3) *E. coli* harboring the pDM2x methyltransferase expression plasmid and grown to an optical density of 0.2 at 600 nm. The culture was divided into 4 equal volumes (250 ml each) and brought to final concentrations of 0, 0.03 mM, 0.50 mM, 04 8.00 mM isopropyl β-D-thiogalactopyranoside. Vigorous shaking at 37°C was continued and samples were collected over 3 hours. Each sample was placed on ice and the cells were pelleted by low speed centrifugation. The pelleted cells were resuspended in buffer A, sonicated, and assayed for methyltransferase activity. The concentration of soluble protein was determined by the modified Lowry assay. A specific activity of 10,000 pmoles/min/mg was used to calculate methyltransferase concentration. Importantly, the methyltransferase was found to comprise up to 20% of soluble total protein in fully active form and, as determined by SDS-PAGE, no methyltransferase polypeptide was found in the insoluble fraction where inclusion bodies are usually found.

A more extensive analysis of methyltransferase production using a concentration of IPTG of 50 μM was then performed. The data in Fig. 5 show that enzyme production occurred rapidly during log-phase growth, resulting in an increasing accumulation of methyltransferase. In Fig. 5, BL21(DE3) cells containing pDM2x were cultured in 1 liter of LB broth at 37°C with shaking at 250 rpm. Induction of expressions begins at time zero by the addition of IPTG to 50 μM. Soluble protein and methyltransferase were assayed as for the times shown in Fig. 4. Only when cell growth began to slow down did the fraction of methyltransferase in the soluble protein fraction level off.

EXPERIMENT 3: HUMAN METHYLTRANSFERASE PURIFICATION

Methyltransferase Purification

Buffer A was used throughout all the preparations and contained 5 mM sodium phosphate, pH 8.0, 5 mM EDTA, 25 μM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM dithiothreitol (DTT), and 10% v/v glycerol.

The cell pellet (14 g) was suspended in 200 ml of buffer A at 4°C. The bacterial cells were lysed by sonication for 2 min on power level 5 at continuous output using a Branson W-350 sonifier with microtip probe. The sonication was done in a 500 ml beaker cooled in an ice-water slurry bath to dissipate heat-buildup and in a

manner ensuring that thorough mixing took place. These sonication settings were optimized in preliminary experiments where samples were lysed at various power levels for various times and assayed for enzyme content by methyltransferase activity. Cell disruption began at power level 3, while activity of the enzyme began to decrease at level 7, presumably due to overheating. The lysed cells were centrifuged at 13,000 g for 15 min and the supernatant saved. 200 ml of buffer A was added to the pelleted debris and this material was again sonicated, pelleted, and the supernatant saved. The supernatants of the two extracts were combined to give a final volume of 390 ml.

Nucleic acids were precipitated by slow addition of protamine sulfate (0.1 volumes (39 ml) of a 4% w/v protamine sulfate solution (Grade X, Sigma)) at 4°C with mixing to the combined supernatant fraction. After mixing for 30 min, the solution was centrifuged at 13,000 g for 15 min and the supernatant saved. The methyltransferase was concentrated and further purified by slowly adding solid ammonium sulfate (Ultrapure, ICN) to 60% saturation (167 g) at 4°C, mixing for 30 min, and centrifuging at 13,000 g for 15 min (Scopes, R.K. (1993) "Protein purification: principles and practice," Springer-Verlag, New York). To remove the ammonium sulfate for the subsequent anion-exchange column purification, the protein pellet was resuspended in 18 ml of buffer A and placed in a 3500 molecular weight cut-off dialysis membrane (Spectrapor 3, Spectrum) and dialyzed three times, every 12 hours, against one liter changes of buffer A.

The final purification step used DEAE-cellulose chromatography under nonequilibrium conditions at room temperature. A 14.7 cm high x 2.5 cm I.D. DE-52 (Whatman) column was equilibrated at a flow rate of 2 ml/min with buffer A as determined by measuring the pH of the eluate. In preparation for sample loading, the column was washed with buffer A with NaCl added to a final concentration of 1 M at a flow rate of 2 ml/min for 1 h, and finally washed with buffer A in the absence of NaCl for 6 hours. 3 ml of the dialyzed ammonium sulfate preparation was then loaded onto the column and washed at 2 ml/min with buffer A for 2 hours. Material bound to the column at this point was eluted by washing with buffer A with 1 M NaCl. 2 minute (4 ml) fractions were collected.

Edman protein sequencing by Dr. Audree Fowler was done at the UCLA Protein Microsequencing Facility using a Porton 2909E sequencer. Electrospray mass spectroscopy was performed at UCLA Molecular and Medical Sciences Mass Spectroscopy Facility by Drs. Ken Conklin and Kym Faull using a Perkin-Elmer Sciex API3 instrument. UV and visible spectroscopy was done on a Hewlett Packard 8452A diode array spectrometer.

The initial batch purification steps used here to enrich the methyltransferase fraction in the lysed bacterial extract is a modification of the procedure used by Fu, *et al.* (Fu, et al. (1991) *J.Biol.Chem.* 266:14562-14572). After lysis of the overexpressing bacteria by sonication, the cellular debris was removed by centrifugation. The nucleic acids remaining in the supernatant were removed by addition of a precipitant, protamine sulfate, and centrifugation. The amount of protamine sulfate required was optimized, and it was found that addition of 0.1 volumes of a 4% solution of protamine sulfate gave a 2.4-fold purification of methyltransferase in essentially quantitative yield (Fig. 6, Table 1). In Fig. 6, various volumes of a 4% w/v protamine sulfate in buffer A were pipetted into 1.0 ml of centrifugation-cleared sonicate supernatant and vortexed. Nucleic acids and other debris were precipitated at 13,000 g for 15 min. Supernatants were assayed for total protein and methyltransferase as

described in Fig. 4. The methyltransferase then was concentrated and purified further by precipitation with ammonium sulfate. Again, conditions for this step in preliminary experiments were optimized. The amount of total protein and methyltransferase precipitated at various percentages of ammonium sulfate saturation is shown in Fig. 7. In Fig. 7, solid ammonium sulfate was added to 1.0 ml samples to give the indicated percent saturation and mixed on a rocking platform for 30 min at 4°C. Protein was pelleted by 13,000 g for 15 min. Pelleted protein was resuspended in an original volume of buffer A and assayed for total protein and methyltransferase as described in Fig. 4. It was found that although the best purification occurred between 50 to 55% saturation, the best compromise between yield and purification occurred at 60% saturation and these conditions were used in the present large-scale purification (Table 1). The pelleted protein was then resuspended in a small volume of buffer A and dialyzed against buffer A to remove the ammonium sulfate.

A rapid, one-step column procedure for purifying the methyltransferase from the dialysate by using DEAE-cellulose anion exchange chromatography was then developed. It was found that application of traditional methodologies where the enzyme was bound to a column in a noninteracting cationic buffer resulted in incomplete purification. However, the use of an interacting anionic buffer under nonequilibrium conditions was found to result in the isocratic elution of homogenous enzyme (Fig. 8). In Fig. 8, protein concentration was determined by measuring the optical density at 280 nm. Electrophoresis was performed using the Laemmli buffer system (Laemmli, U.K. (1970) *Nature* 227:680-5) using a 12% Duracryl (Millipore) separating gel. Polypeptides were visualized by rapid silver staining (Blum, et al. (1987) *Electrophoresis* 8:93-99.). Fractions were analyzed on two mini gels: 1μL Sonicate and Load, 10μL of 10-21 on gel #1 and; 15 μL of LMWS/100, 10μL fractions 22-25, 27,29,31,33,35, 1μL of fractions 76-80 on gel #2. Under these conditions, the methyltransferase elutes as a narrow spike of enzyme centered on fraction 18 and as a broader peak from fractions 21 to 40. The methyltransferase in these two peaks appears to be identical. Both peaks are characterized by predominant 25 kDa polypeptides in SDS gel analysis (Fig. 9). In Fig. 9, 1 ml fractions of purified enzyme were dialyzed against 20 mM sodium citrate at the pH values indicated and the fractions were concentrated by ultrafiltration using Centricon-10 micro concentrators (Amicon, Beverly, MA). The first peak shows a low level of contamination (less than 5% for fraction 18) by other polypeptides while the second, broader peak shows no other protein contamination. The specific activity of the methyltransferase is identical in both peaks, and their weight by mass spectroscopy is also identical (see below). It appears that the partial resolution of these peaks is due to the nonequilibrium nature of the chromatography as well as the amount of protein loaded. For example, a smaller loading of dialysate results in only a single broad peak of homogeneous enzyme eluting further from the void volume, and a larger loading results in a narrow peak with a higher concentration of enzyme (with minor contaminating polypeptides) near fraction 18 (results not shown). If the chromatography is performed where the column is fully equilibrated before loading, then the methyltransferase is only slightly retarded and elutes close to the void volume along with small amounts of contaminating polypeptides.

Table 1 summarizes the purification steps involved in obtaining large amounts of homogenous methyltransferase. It shows that the largest loss in yield occurs during the ammonium sulfate precipitation step; the reason for this is not understood. Overall, 17 mg of enzyme from each 1.7 liters of the original broth culture

at a specific activity of 10,000 pmoles/min/mg, comparable to that found for the purified human erythrocyte enzyme (Gilbert, et al. (1988) *Biochemistry* 27:5227-5233, Ingrosso, et al. (1989) *J.Biol.Chem.* 264:20131-20139), was obtained. In this preparation, the column chromatography step using only one-sixth of the total preparation (Table 1) was performed. Repeated cycles of DEAE-cellulose chromatography can thus be used to readily generate additional homogenous enzyme.

It was found that the purified recombinant enzyme is stable at room temperature for up to 2 months and repeated freeze-thaw cycles have no effect on activity. Furthermore, it was found that the methyltransferase can be heated for up to 30 min at 50°C with no loss of activity.

TABLE 1

Purification of human recombinant protein-L-isoaspartate (D-aspartate) O-methyltransferase from *E. coli* Cells from 10l of a culture in LB broth at an optical density of 1.6 at 600 nm.

Sample	Volume	Total Protein	Total Activity	Specific Activity	Yield	Purification
	mL	mg	pmol/min	pmol/min/mg	%	-fold
Sonicate	400	3680.0	4800000	1304	100	1.0
Cleared Supernatant	390	1358.0	3471000	2556	72	2.0
0.4% Protamine Sulfate	428	562.4	3474900	6178	72	4.7
60% Ammonium Sulfate	18	291.6	1602000	5494	33	4.2
Dialysate	18	146.0	1062000	7275	22	5.6
DEAE Pool - Fractions 16-70[a]	220	17.2	171500	10000	21[b]	8.0

a This represents the loading of 3 ml of dialysate (or 1/6 of the total preparation) - the protein and methyltransferase content was determined for individual fractions and combined to give the values reported here.

b Corrected for loading of 3 ml of the 18 ml of dialysate.

Enzyme Concentration

The availability of a concentrated form of the enzyme is important for several uses including x-ray structure determination. It was found that successful concentration of the methyltransferase was extremely pH dependent. Contrary to typical protein solubility characteristics (Scopes, R.K. (1993) "Protein purification: principles and practice," Springer-Verlag, New York.), concentration of this enzyme occurred most readily near its isoelectric point of 5.9 pH units (M. Redinbo, personal communication) (Fig. 8).

Characteristics of Purified Recombinant Methyltransferase

N-terminal sequencing of the purified methyltransferase was performed by automated Edman degradation analysis. The experimentally-determined sequence of the first twenty residues was exactly that predicted from the cDNA with the removal of the initiator methionine as found in the human enzyme (Ingrosso, et al.(1989) *J.Biol.Chem.* 264:20131-20139) (Table 2). It was found no evidence for a blocked amino terminus, such as the acetylated alanine residue present in the human enzyme (Ingrosso, et al.(1989) *J.Biol.Chem.* 264:20131-20139).

The molecular weight of the purified enzyme was measured at 24,551 ±3 Da by electrospray mass spectroscopy (Fig. 10 (A)). In Fig. 10 (A), a portion of the spectrum of material purified was described above using dithiothreitol as the reductant in buffer A is shown. No other peaks were discerned at other molecular weights. This average value matched exactly the predicted value of 24,551 Da of the unmodified product encoded by the cDNA sequence. Preliminary attempts at purification using β-mercaptoethanol instead of dithiothreitol as the reducing agent produced an enzyme that had 1 or 2 adducts of β-mercaptoethanol as indicated by the 2 additional minor forms at higher molecular weights of 24,627 and 24,702 Da, respectively (Fig. 10(B)). In Fig. 10 (B), a portion of the spectrum of material purified when 15mM β-mercaptoethanol was substituted for 0.1 mM ditiothreitol in buffer A. The peak at a mass of 24,627 represents an adduct with 1 β-mercaptoethanol molecule and the smaller peak at 24,704 represents the adduct with 2 β-mercaptoethanol molecules.

The structure of the protein product by on-line liquid chromatography electrospray mass spectral analysis of tryptic and cyanogen bromide fragments were also directly confirmed. With the exception of an insoluble hydrophobic core corresponding to residues 37 to 143, the mass of all detected species corresponded to predicted fragments.

For direct spectrophotometric concentration determination of homogenous methyltransferase, the extinction coefficient of the enzyme from amino acid composition to correspond to 1.12 mg/ml for 1 A_{280nm} (Mach, et al.(1992) *Anal.Biochem.* 200:74-80) was calculated. This value was verified by the trichloroacetic acid-Lowry method using bovine serum albumin as a standard (Chang, (1992) *Anal.Biochem.* 205:22-26). The UV spectrum of the homogenous methyltransferase in fraction 29 from the DEAE column (Fig. 8) is shown in Fig. 11.

TABLE 2

N-terminal Edman sequencing analysis of purified human recombinant protein-L-isoaspartate (D-aspartate) O-methyltransferase from *E. coli*. 150 pmoles of methyltransferase in 10 μL of 10mM neutral ammonium bicarbonate were loaded.

Cycle	Residue Identified	pmol	Cycle	Residue Identified	pmol
1	Ala	68	11	Glu	7
2	Trp	25	12	Leu	10
3	Lys	12	13	Ile	6
4	Ser	8	14	His	8
5	Gly	19	15	Asn	6
6	Gly	23	16	Leu	9
7	Ala	20	17	Arg	9
8	Ser	6	18	Lys	4
9	His	12	19	Asn	6
10	Ser	6	20	Gly	13

N-terminal sequence encoded by cDNA = MAWKSGGASHSELIHNLRKWG

EXPERIMENT 4: IDENTIFICATION OF METHYLTRANSFERASE IN PLANTS

Biological Materials

Fresh carrots, yellow corn, Romaine lettuce, green peas, white potatoes, spinach, cherry tomatoes, and alfalfa were purchased at a local distributor. Alfalfa seeds and raw wheat germ were from Rainbow Acres, Inc. (Los Angeles, CA), while soybean seeds were from Arrowhead Mills, Inc. (Hereford, TX). Winter wheat (*Triticium aestivum* cultivar Augusta) seeds were provided by Dr. Robert Forsberg of the University of Wisconsin (Madison, WI). Danver Half Long carrot seeds, Golden Jubilee corn seeds, Romaine lettuce seeds, sugar snap pea seeds, New Zealand spinach seeds, and Bonny Best tomato seeds were from the Chas. H. Lilly Co. (Portland, OR). A cytosolic fraction of *Chlamydomonas reinhardtii* (Wt strain 2137) was provided by Drs. Gregg Howe and Sabeeha Merchant of the University of California at Los Angeles (Howe & Merchant, 1992).

Preparation of Plant Cytosol

Crude cytosol was extracted from the plant tissues by homogenization using a mortar and pestle. In a chilled mortar, liquid nitrogen was poured over plant tissue (typically, 20 g of fresh tissue or 5 g of seeds) until the tissue was completely frozen. To remove undesirable polyphenol oxidases potentially released from the tissue upon homogenization, 3 g of hydrated PVPP (polyvinyl-polypyrrolidone) (Loomis, et al.(1966) Phytochemistry 5, 423-438) was thoroughly mixed with the frozen tissue before the tissue was ground with a pestle. Extraction buffer (20 mL of 100 mM HEPES, pH 7.5, 10 mM 2-mercaptoethanol,

1 μm leupeptin, 1 mM PMSF, 10 mM sodium hydrosulfite, and 10 mM sodium metabisulfite at 4°C) was added to the mortar, and the slurry was ground further. The resulting crude homogenate was pressed through four layers of cheesecloth and then centrifuged at 2200 g for 30 min at 4°C to remove the insoluble PVPP and undisrupted plant material. The resulting supernatant was centrifuged further at 172200xg for 50 min at 4°C and then filtered through two layers of Miracloth (Calbiochem, San Diego, CA) to remove the floating lipid layer. This fraction, identified as crude cytosol, was stored at -80°C and utilized as the source of methyltransferase.

Methylation Assay

Methyltransferase activity was identified using a vapor-phase diffusion assay that quantitates the number of radiolabeled methyl groups transferred for S-adenosyl-L-[*methyl*-^{14}C] methionine to a peptide substrate by quantitating the release of [^{14}C] methanol resulting from the hydrolysis of base-labile methyl esters. In a total reaction volume of 40 μL, 12 μL of enzyme preparation was incubated with 10 μM S-adenosyl-l-[*methyl*-^{14}C]methionine (ICN Biomedicals, 50 mCi/mmol), 500 μM peptide substrate, and 0.33 M HEPES, pH 7.5. Peptide substrates [VYP-(L-isoAsp)-HA (SEQ ID NO:15), KASA-(L-isoAsp)-LAKY (SEQ ID NO:16), AA-(L-isoAsp)-F-NH_2 (SEQ ID NO:17), VYG-(D-Asp)-PA (SEQ ID NO:18), and KASA-(D-Asp)-LAKY (SEQ ID NO:19)] were synthesized by Dr. Janis Young at the UCLA Peptide Synthesis Facility and characterized as described previously (Lowenson, et al.(1991b) *J.Biol.Chem.* 266, 19396-19406, (1992) *J.Biol.Chem.* 267, 5985-5995). Alternatively, during the purification of the wheat germ methyltransferase (see below), samples were assayed in buffer containing a final concentration of 0.2 M sodium citrate, pH 6.0. In either case, incubations were performed at 25°C for 60 min. Each reaction was then quenched with 40 μL of 0.2 M NaOH and 1% (w/v) SDS and vortexed, and a 60-μL aliquot was spotted onto a 1.5 x 8 cm pleated filter paper (Bio-Rad no. 165-090) and placed in the neck of a 20-mL scintillation vial containing 5 mL of Bio-Safe II (RPI, Mount Prospect, IL) counting fluor. The vials were capped, and [^{14}C]methanol was allowed to diffuse from the paper through the vapor phase to the fluor, while the nonvolatile ^{14}C radioactivity remained on the paper. After 2 h at room temperature, the paper was removed from the necks of the vials and the vials were counted.

Protein Determination

A modification of the Lowry procedure (Bailey (1967) Techniques in Protein Chemistry,Elsevier Publishing Co.,New York) was used to determine the concentration of protein after precipitation with 1 mL of 10% (w/v) trichloroacetic acid.

Identification of L-Isoaspartyl Methyltransferase in Plants

Representatives from both classes of angiosperms as well as a green alga were surveyed for the presence of L-isoaspartyl methyltransferase. Crude cytosol was isolated from different types of plant material and then assayed for methyltransferase activity using the L-isoaspartyl-containing peptide, VYP-(isoAsp)-HA (SEQ ID NO:15), which has been shown to be an excellent peptide substrate for the human enthryocyte methyltransferase (K_m=0.29 μM; Lowenson & Clarke, 1991). Endogenous cytosolic

oligopeptides are also potential methyl acceptors; therefore, parallel experiments were conducted in the presence and absence of the peptide substrate (Table 3). Peptide dependent L-isoaspartyl methyltransferase was found in the vegetative cells of the green alga *C. reinhardtii*, demonstrating its presence in at least one species in the Kingdom Protista. In the Kingdom Plantae, methyltransferase activity was detected in both classes of the angiosperms, the monocots and the dicots. The level of activity in different tissues varied considerably. Of the species assayed, the highest specific activity of the methyltransferase was found in wheat embryos. In contrast, almost no detectable L-isoaspartyl peptide-specific methyltransferase activity was found in the leaves of lettuce or the fruits of tomato. Significantly, high levels of methyltransferase activity were found in the seeds of all plants assayed, including corn, alfalfa, lettuce, pea, spinach, and tomato, as well as in the roots of carrots and potatoes. The specific activity of the enzyme in plant seeds (0.66 - 14.0 pmol/min/mg) is comparable to the levels found in *E. coli* (1-2.5 pmol/min/mg; Fu, et al., 1991) and human erythrocytes (1.9 - 9.4 pmol/min/mg; Ota, et al., 1988; Gilbert, et al. 1988).

TABLE 3

OCCURRENCE OF L-ISOASPARTYL METHYLTRANSFERASE ACTIVITY IN THE SOLUBLE FRACTION OF PLANTS

Species	Plant Material	methyltransferase activity* (pmol/min/mg of protein) Endogenous Substrates	L-isoAsp peptide
Green alga			
C. reinhardtii	vegetative cells**	0.15±0.00	0.43±0.01
Monocots			
corn	fresh kernels	0.30±0.04	1.46±0.11
	dry kernels	0.71±0.03	6.86±0.42
wheat	embryos (germ)	0.33±0.05	14.0±0.14
	kernels	0.39±0.01	4.36±0.09
Dicots			
alfalfa	seedlings	0.35±0.01	0.47±0.02
	seeds	0.34±0.03	3.42±0.25
carrots	roots	0.96±0.07	2.64±0.28
	seeds	0.44±0.03	1.37±0.04
lettuce	leaves	0.27±0.00	0.29±0.01
	seeds	0.14±0.01	0.66±0.01
pea	fresh seeds	0.24±0.04	1.31±0.05
	dry seeds	0.12±0.00	1.79±0.10
potato	roots	0.19±0.01	1.04±0.00
soybean	seeds	0.12±0.00	0.69±0.03
spinach	leaves	0.22±0.01	1.10±0.03
	seeds	2.16±0.12	2.60±0.05
tomato	fruit	2.90±0.16	3.03±0.17
	seeds	1.17±0.02	8.07±0.15

Methylation assays were performed in triplicate.

EXPERIMENT 5: PURIFICATION OF METHYLTRANSFAREASE FROM WHEAT GERM

Preparation of Wheat Germ Cytosol for Enzyme Purification

Raw wheat germ (150 g) was suspended in 750 mL of buffer (20 mM sodium borate (pH 9.3), 5 mM EDTA, 2.4 mM 2-mercaptoethanol, and 25 mM NaCl) and stirred for 30 min at 4°C. The slurry was then squeezed through four layers of cheesecloth, and the resulting crude homogenate (585 mL) was centrifuged at 7000xg for 60 min at 4°C to remove membrane and cell debris. The supernatant (520 mL) was poured through two layers of Miracloth to filter the floating lipid layer.

Purification of L-Isoaspartyl Methyltransferase from Wheat Germ

The present purification strategy was based on the partial purification of the protein carboxyl methyltransferase reported by Trivedi, et al. (Trivedi, et al.(1982) *Eur.J.Biochem.* 128, 349-354). Referring to Fig. 12, crude wheat germ cytosol (515 mL, 30 mg of protein/mL) was loaded onto a DE-52 (Whatman)

column (9 cm diameter x 13 cm resin height, 827 mL) which was previously equilibrated at 4°C with buffer (20 mM sodium borate (pH 9.3), 5 mM EDTA, 2.4 mM 2-mercaptoethanol, and 25 mM NaCl). Two-minute fractions were collected at an average flow rate of 8-10 mL/min. The loaded column was washed isocratically with 1 L of buffer followed by a 6-L gradient of 25-200 mM NaCl in the above buffer. The protein profile and the NaCl gradient were monitored by measuring absorbance at 280 nm and conductivity, respectively, in the corresponding fractions. Every fifth fraction was assayed for L-isoaspartyl methyltransferase using VYP-(L-isoAsp)-HA (SEQ ID NO:15) as the peptide substrate. One peak of methyltransferase activity was pooled (fractions 80-110,600 mL, see brackets) and further purified by reverse ammonium sulfate gradient solubilization as described by King (Biochemistry 11, 367-371, 1972). The pH of the DE-52 pooled material was adjusted to 8.38 with 20 mL of 1 M Tris-HCl, pH 7.97. 15.62 g of Celite 545 (Baker Analyzed Reagent, 11 g of Celite/1 g of protein) was then added with stirring to 80% saturation (56.1 g of ammonium sulfate/100 mL initial volume) in a 30-min period at room temperature, and then stirring was continued for an additional 45 min. This Celite mixture containing precipitated cytosolic proteins was poured into a 3 cm diameter x 19 cm column and packed with the aid of a peristalic pump at room temperature. The column was washed isocratically with 150 mL (approximately two column vol) of 80% saturated ammonium sulfate solution containing 0.05 M Tris-HCl (pH 7.97) and eluted with a 550-mL linear gradient decreasing from 80 to 0% saturation in ammonium sulfate. The flow rate of the gradient was approximately 0.6 mL/min, and 7.5-min fractions were collected. The percent of ammonium sulfate and protein in the corresponding fractions was determined by conductance and absorbance at 280 nm, respectively. Every second fraction was assayed for L-isoaspartyl methyltransferase using VYP-(L-isoAsp)-HA (SEQ ID NO:15) as the peptide substrate. Fractions (65-74), containing the highest specific activity of the methyltransferase, were pooled (95 mL, see brackets) and subsequently purified on a Sephacryl S-200 (Sigma) gel filtration column (2 cm diameter x 77.5 cm resin high, 243 mL). Buffer containing 20 mM Tris-acetate (pH 7.0), 0.2 mM EDTA, 15 mM 2-mercaptoethanol, and 10 mM NaCl was used to equilibrate and run the column at 4°C. The flow rate of the column was maintained at 0.12 mL/min and 30-min fractions were collected. Every second fraction was assayed for L-isoaspartyl methyltransferase using VYP-(L-isoAsp)-HA as the peptide substrate. Absorbance at 280 nm was measured to determine the protein concentration of these fractions. Purified wheat germ L-isoaspartyl methyltransferase consistently eluted in one or two fractions, roughly corresponding to a fraction volume of 134-139 mL (see arrow Fig. 12C).

Surprisingly, the L-isoaspartyl methyltransferase eluted in a highly purified state in a fraction nearly corresponding to the total volume of the column. This success in obtaining a highly purified enzyme preparation from the Sephacryl S-200 gel filtration column was attributed to this unusual absorption phenomenon.

The overall purification of the L-isoaspartyl methyltransferase from wheat germ is summarized in Table 4, and the typical polypeptide composition corresponding to each step in the purification is shown

in Fig. 13. Referring to Fig. 13, active fractions containing methyltransferase from each purification step were analyzed by SDS-PAGE using the buffer system described by Laemmli (Laemmli (1970) *Nature* 227, 600-685). Protein fractions were mixed in a ratio of 2:1 (v/v) with sample buffer [160 mM Tris-HCl (pH 6.8), 6.0% (w/v) SDS, 2.1 M 2-mercaptoethanol, 35.5% (v/v) glycerol, and 0.004% (w/v) bromophenol blue] and boiled for 3 min. These fractions were electrophoresed in a 12.5% (w/v) acrylamide/0.43% (w/v) N,N-methylenebisacrylamide separating gel. Gels were stained in Coomassie brilliant blue. The molecular mass standards (Bio-Rad) included phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14 kDa). The samples analyzed were crude wheat germ homogenate (lane A), filtered crude cytosol (lane B), fractions 80-100 from the DEAE-cellulose column (lane C), reactions 65-74 from the reverse ammonium sulfate gradient solubilization step (lane D), and fraction 39 from the Sephacryl S-200 column (lane E). The position of the methyltransferase polypeptide (MT) is indicated at the right with an arrow (Fig. 13).

TABLE 4

PURIFICATION OF L-ISOASPARTYL METHYLTRANSFERASE FROM WHEAT GERM CYTOSOL

sample	volume (mL)	total protein (mg)	total activity* (pmol/min)	% recovery	specific activity (pmol/min/mg)	purification
crude homogenate	585	19012	69205	100	3.64	1.0
crude cytosol (7000 g)	520	15600	57616	83.3	3.69	1.0
DEAE-cellulose	600	600	52020	75.2	86.7	23.8
reverse ammonium sulfate gradient solubilization	45.3	24.9	10140	14.7	436.4	119.9
Sephacryl S-200	15.0	1.04	5025	7.3	4855.1	1333

* Measured at pH 6.0 using 500 μM VYP-(isoAsp)-HA as a methyl acceptor.

Mono Q Anion Exchange Chromatography

Fractions containing L-isoaspartyl methyltransferase from several Sephacryl S-200 gel filtration columns were pooled and then dialyzed (Spectropor, cutoff 3500 Da) in buffer A. Dialyzed methyltransferase (0.6 mg of protein) was fractionated on a Mono Q HR 5/5 anion exchange (Pharmacia) column (5 mm diameter x 50 mm resin height, 1 mL) previously equilibrated with buffer A. One-minute fractions were collected at a flow rate of 0.5 mL/min. The loaded column was washed isocratically with buffer A for 15 min followed by a linear gradient of 0 to 100% buffer B (20 mM Tris-acetate (pH 7.0),

0.2 mM EDTA, 15 mM 2-mercaptoethanol, 10% glycerol, and 1 M sodium acetate) over 60 min. The column effluent was monitored at 280 nm. Typically methyltransferase activity was detected in fractions 43-44. Fractions containing active methyltransferase were pooled and used for enzymological studies.

The calculated molecular mass of the major polypeptide determined by SDS-polyacrylamide slab gel electrophoresis was 28,000 Da. It was demonstrated that this polypeptide corresponds to the L-isoaspartyl methyltransferase by renaturing individual gel slices in the presence of Triton X-100 as described by Clarke (Clarke (1981) Biochim.Biophys.Acta 670, 195-202). The purity of this preparation was estimated to be 86% from densitometry of the Coomassie-stained gel. The remaining minor polypeptide contaminants could be removed by an additional chromatography step. Dialyzed methyltransferase was loaded onto a Mono Q anion exchange column and eluted with a linear gradient of 0-1 M sodium acetate. Active methyltransferase eluted at approximately 0.5 M sodium acetate.

Characterization of L-Isoaspartyl Methyltransferase from Wheat Germ

Methyltransferase purified through the Mono Q step [12,500 pmol/min/mg at pH 7.5 with the VYP-(isoAsp)-HA peptide as the substrate] was used to study the specificity of the wheat germ enzyme. Like the *E. coli* and human erythrocyte methyltransferases, the wheat germ enzyme efficiently methylates L-isoaspartyl residues in synthetic peptides.

EXPERIMENT 6: AMINO ACID SEQUENCE OF PURIFIED WHEAT GERM METHYLTRANSFERASE

Reverse-Phase HPLC

Homogeneous methyltransferase suitable for sequence analysis was obtained by reverse-phase HPLC (high-performance liquid chromatography) of the enzyme purified through the Sephacryl S-200 step. Fractions were loaded onto a Vydac C-4 column (1 cm i.d. x 25 cm, 300-Å pore, 5 μm spherical silica support) equilibrated with 65% solvent B and eluted with a linear gradient of 65-80% solvent B over 45 min at 3.0 mL/min flow rate, where solvent A is 0.1% trifloroacetic acid in water (w/v) and solvent B is 0.1% trifluoroacetic acid in 99% methanol/0.9% water (w/v/v). The column effluent was monitored at 280 nm as 1-min fractions were collected. Volatile reagents were removed from the fractions in a Savant Speedvac apparatus, and then these fractions were subjected to SDS-PAGE and silver staining (Jones (1990) in Current Protocols in Molecular Biology Suppl. 11, John Wiley and Sons, New York). The wheat germ methyltransferase eluted at about 40 min as a single polypeptide band with an apparent molecular mass of 28,000 Da.

Amino Acid Sequence Determination by Tryptic and Staphylococcus aureus V8 Protease Mapping

Homogeneous methyltransferase suitable for amino acid sequence analysis was obtained by reverse-phase HPLC analysis as described above. This material was digested with trypsin and *Staphylococcus aureus* V8 protease, and the resulting peptides were recovered by reverse-phase HPLC using a Vydac C-18 column. N-terminal Edman sequencing was then performed on these peptides. The partial peptide sequence data obtained were used to generate oligonucleotide probes and to confirm the presence of polymorphisms and/or multiple genes (see EXPERIMENT 7 below).

EXPERIMENT 7: cDNA CODING FOR WHEAT METHYLTRANSFERASE

Synthetic Oligonucleotide Probes

Oligonucleotide probes were synthesized using β-cyanoethyl N,N-diisopropylphosphoramidite chemistry in a Gene Assembler Plus DNA synthesizer (Pharmacia LKB Biotechnology). An oligonucleotide representing the T7 promoter of the pBluescript SK± phagemid, T7 [DMT-TAATACGACTCACTATAGGG] (SEQ ID NO:11), and three degenerate oligonucleotides, MB1 [TCTGG(G/A)AT(G/A)TG(C/T)TC(G/A)ATNCCCAT] (SEQ ID NO:12), MB3 containing an *Eco*RI linker [CTCGAATTCTA(C/T)(C/T)T-NAA(G/A)CA(G/A)TA(C/T)GGNGT] (SEQ ID NO:13), and MB4 containing a *Hind*III linker [TCAAAGCTTTT(G/A)TC(T/G/A)ATNAC(C/T)TGNAG] (SEQ ID NO:14), were synthesized for use as probes and as primers in PCR amplification of a wheat cDNA library (described below). The primers were purified by size exclusion chromatography using Bio-Spin 6 columns (Bio-Rad).

Isolation of a cDNA Clone for L-Isoaspartyl Methyltransferase From Wheat

Degenerate oligonucleotides were synthesized on the basis of the partial amino acid sequence data (see above) and then used to amplify a region of the methyltransferase cDNA from a wheat cDNA library constructed with poly $(A)^+$ RNA isolated from 48-h-etiolated wheat seedlings (Hatfield, et al.(1990) *J.Biol.Chem.* 265, 15813-15817). An 850-bp PCR product was amplified using a 384-fold degenerate oligonucleotide, MB3, representing the nucleic acid sequence at the 5'-region of the methyltransferase cDNA (corresponding to the peptide YLKQYGV) and a primer encoding the T7 promoter of the pBluescript vector. The identity of the 850-bp PCR product was verified by Southern hybridization using a 64-fold degenerate oligonucleotide, MB1, representing the nucleic acid sequence in the middle region of the methyltransferase cDNA (corresponding to the peptide GIEHIPE). In order to obtain a PCR product containing the methyltransferase cDNA without the pBluescript vector sequence, the 850-bp PCR product was used as a template in a PCR reaction with the MB3 primer and a 288-fold degenerate oligonucleotide, MB4, representing the nucleic acid sequence at the 3'-region of the methyltransferase cDNA (corresponding to the peptide LQVIDK). PCR amplification produced a 600-bp fragment containing only the methyltransferase cDNA sequence, as determined by PCR dideoxy chain-termination sequencing. Screening of the wheat cDNA library with this 600-bp product resulted in the isolation of one positive plaque which was rescued to a pBluescript phagemid in *E. coli* XL-1 pUC19 to give the pMBM1 plasmid, which was then used to transform *E. coli* DH5α cells.

DNA Sequence of the Gene Encoding L-Isoaspartyl Methyltransferase from Wheat

The DNA sequence of the 952-bp cDNA insert in the plasmid pMBM1 was determined using the sequencing strategy shown in Fig. 14. Referring to Fig. 14, both strands of the pMBM1 clone containing the wheat methyltransferase cDNA insert (a 952-bp *Eco*RI fragment) were sequenced by dideoxy chain-termination sequencing. Oligonucleotides were synthesized using the sequence of a PCR product containing 600 bp of the wheat methyltransferase cDNA and then used as primers to sequence the 952-bp fragment as shown in the sequencing strategy in this figure. The DNA sequence of the methyltransferase cDNA and

its deduced amino acid sequence are indicated as SEQ ID NOs:5 and 6, respectively. Referring to SEQ ID NOs:5 and 6, the sequence of the coding strand of the 944-bp insert of the plasmid pMBM1 is shown without the terminal *Eco*RI linkers (GGAATTCC) that were added to the cDNA library. The 690-bp methyltransferase cDNA initiates at the ATG codon at position 118 and terminates at the TGA codon at position 808. The calculated molecular weight of the 230 amino acid polypeptide deduced for the 690-bp open reading frame is 24,710. In contrast, purified methyltransferase migrated as a 28,000-Da polypeptide as determined by SDS-PAGE (Fig. 13).

EXPERIMENT 8: COMPARISON OF SEQUENCED PEPTIDE FRAGMENTS AND PREDICTED SEQUENCE

Comparison of Sequenced Peptide Fragments of L-isoaspartyl methyltransferase from Wheat Germ and Its Predicted Amino Acid Sequence from pMBM1

Interestingly, discrepancies at 12 sites between the predicted amino acid sequence of the wheat cDNA and the sequence of the peptide fragments of the wheat germ L-isoaspartyl methyltransferase were found (Fig. 15). Referring to Fig. 15, following digestion with trypsin (T) and *S. aureus* V8 protease (V), peptides were recovered by reverse-phase HPLC and sequenced by automated Edman degradation. Peptide sequences of fragments numbered in the order of elution are shown by lines in comparison to the deduced cDNA sequence. The presence of a space indicates that unambiguous identification of the amino acid residue could not be made in this cycle. Additional residues identified in a particular cycle are indicated in parentheses above. Residues above in brackets denote an amino acid substitution at this position where no evidence was found for the cDNA-encoded residue. As a whole, the amino acid sequence of purified wheat enzyme is considered to be indicated in SEQ ID NO:7 with the following exceptions. At position 41, A was found in T8 and V13; N was found in T9. At position 52, I was fond in T8, V14, and V17. At position 54, only L was found in V17. At position 156, A was found in T6 and V8; V was found in T7 and V9. In six of these positions, the experimentally determined amino acid sequence data clearly show the presence of an amino acid not encoded by the cDNA. At the other six positions, residues in addition to the encoded residue were identified by Edman sequencing. These results are consistent with the hexaploid nature of this species of wheat, where the three diploid genomes (AABBDD) can contain alleles with variant sequences, leading to the production of variant gene products (Peumans, et al.(1982) *Planta* 154, 562-567; Wright, et al.(1989) *J.Mol.Evol.* 28, 327-336). Most of the amino acid changes are located outside of the three highly conserved regions shared among methyltransferases. It is interesting to speculate that these amino acid differences can result in enzymes with slightly different methyl acceptor specificities, which would give the cell the ability to recognize and potentially repair a wider range of damaged proteins. According to the present purification method, these enzyme variants can be purified as well.

EXPERIMENT 9: SKIN TREATMENT USING METHYLTRANSFERASE

Purified methyltransferase (either isolated from plant sources or as a recombinant human enzyme produced in bacteria) and S-adenosyl-L-methionine are mixed to final concentrations of 0.01% and

0.00004% respectively in a water-based cream containing glycerin and mineral oil. A skin or hair cream is formulated using water, glycerin, cetearyl alcohol, palm oil glyceride, Ceteareth-20, mineral oil, petrolatum, sorbitol, avocado oil, DMSO (or other carrier molecules), steric acid, alantoin, squalane, methylparaben, Sodium Carbomer 941, recombinant human L-isoaspartyl/D-aspartyl methyltransferase (or plant methyltransferase), propylparaben, S-adenosyl-L-methionine, Quaternium-15, fragrance, FD&C Yellow No.5, and FD&C Red No.4. A skin mist ("Methylmist") is formulated using water, glycerin, cetearyl alcohol, DMSO (or other carrier molecules), citric acid, recombinant human L-isoaspartyl/D-aspartyl methyltransferase (or plant methyltransferase), S-adenosyl-L-methionine, Quaternium-15, and fragrance. A transdermal skin patch is formulated using the same ingredients as the above skin mist. These preparations can be directly applied to the skin. After cleaning the skin with soap and water, the methyltransferase skin cream (a), mist (b) and patch (c) are used by the following methods respectively: (a) applying a dab (0.5ml) of a viscous formulation to a central spot and spreading over an area of about 100 cm^2 by rubbing with small circular motions directly by hand with or without latex gloves and/or with or without applicants such as towels and tissues; (b) using a less viscous formulation ("MethylMist") and spraying on with a spray bottle or atomizer in which the residue can be wiped into the skin as described above for the skin cream; and (c) using the same formulation as "MethylMist" but enclosing in a patch that adheres to skin and delivers the substance through micro-pores in the patch directly to the skin.

EXPERIMENT 10: OTHER MEDICAL TREATMENT USING METHYLTRANSFERASE

An injectable or topical preparation (pH – 7.3) for treatment of the eye (e.g.,for preventing cataracts). Injectable preparations for the brain (e.g., for preventing plaque formation) or the blood stream (e.g., for maintaining flexibility) contains 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 25 mM recombinant human L-isoaspartyl/D-aspartyl methyltransferase (saturation) or 1-3 μM to match S-adenosyl-L-methionine concentrations, 30 μM S-adenosyl-methionine, and 0.22 μM sterile filtered. For time-release delivery systems, the injectable preparation can be packaged in carrier liposomes or microporous structures. These preparations can be directly injected to the eye, brain or blood stream.

EXPERIMENT 11: DIAGNOSIS USING METHYLTRANSFERASE

A sample of cerebrospinal fluid (0.01 ml) diluted in 0.2 M sodium citrate buffer, pH 6.0 is mixed with 0.03 ml of a mixture of S-adenosyl-[^{14}C-methyl]-L-methionine (100 cpm/pmol) and purified methyltransferase (from plant sources or human recombinant) in the same buffer. The latter mixture contains 400 pmol of radiolabelled S-adenosylmethionine and 10 micrograms of purified methyltransferase. The mixture is incubated for 15 min at 37 °C and then 0.04 ml of a solution of 0.1% sodium dodecyl sulfate, 0.2 M sodium hydroxide is added. This mixture is then applied to a piece of thick filter paper and is suspended in a plastic vial containing 7 ml of liquid scintillation cocktail. After 2 hours at room temperature, the paper is removed and the vial counted. The radioactivity is proportional to the amount of damaged L-isoaspartyl and D-aspartyl residues in the cerebrospinal fluid.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

(ii) TITLE OF THE INVENTION: PRODUCTION AND USE OF HUMAN AND PLANT METHYLTRANSFERASES

(iii) NUMBER OF SEQUENCES: 19

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Knobbe, Martens, Olson and Bear
(B) STREET: 620 Newport Center Drive 16th Floor
(C) CITY: Newport Beach
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 92660

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Altman, Daniel E
(B) REGISTRATION NUMBER: 34,115
(C) REFERENCE/DOCKET NUMBER: UCLA010.001A

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 714-760-0404
(B) TELEFAX: 714-760-9502
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 681 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGGCCTGGA AATCCGGCGG CGCCAGCCAC TCGGAGCTAA TCCACAATCT CCGCAAAAAT 60
GGAATCHTNA AGACAGATAA AGTATTTGAA GTGATGCTGG CTACAGACCG CTCCCACTAT 120
GCAAAATGTA ACCCATACAT GGATTCTCCA CAATCAATAG GTTTCCAAGC AACAATCAGT 180

GCTCCACACA TGCATGCATA TGCGCTAGAA CTTCTATTTG ATCAGTTGCA TGAAGGAGCT 240
AAAGCTCTTG ATGTAGGATC TGGAAGTGGA ATCCTTACTG CATGTTTTGC ACGTATGGTT 300
GGATGTACTG GAAAAGTCAT AGGAATTGAT CACATTAAAG AGCTAGTAGA TGACTCARTN 360
AATAATGTCA GGAAGGACGA TCCAACACTT CTGTCTTCAG GGAGAGTACA GCTTGTTGTG 420
GGGGATGGAA GAATGGGATA TGCTGAAGAA GCCCCTTATG ATGCCATTCA TGTGGGAGCT 480
GCAGCCCCTG TTGTACCCCA GGCGCTAATA GATCAGTTAA AGCCCGGAGG AAGATTGATA 540
TTGCCTGTTG GTCCTGCAGG CGGAAACCAA ATGTTGGAGC AGTATGACAA GCTACAAGAT 600
GGCAGCATCA AAATGMRNCC TCTGATGGGG GTGATATACG TGCCTTTAAC AGATAAAGAA 660
AAGCAGTGGT CCAGGTGGAA G 681

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 684 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGCCTGGA AATCCGGCGG CGCCAGCCAC TCGGAGCTAA TCCACAATCT CCGCAAAAAT 60
GGAATCHTNA AGACAGATAA AGTATTTGAA GTGATGCTGG CTACAGACCG CTCCCACTAT 120
GCAAAATGTA ACCCATACAT GGATTCTCCA CAATCAATAG GTTTCCAAGC AACAATCAGT 180
GCTCCACACA TGCATGCATA TGCGCTAGAA CTTCTATTTG ATCAGTTGCA TGAAGGAGCT 240
AAAGCTCTTG ATGTAGGATC TGGAAGTGGA ATCCTTACTG CATGTTTTGC ACGTATGGTT 300
GGATGTACTG GAAAAGTCAT AGGAATTGAT CACATTAAAG AGCTAGTAGA TGACTCARTN 360
AATAATGTCA GGAAGGACGA TCCAACACTT CTGTCTTCAG GGAGAGTACA GCTTGTTGTG 420
GGGGATGGAA GAATGGGATA TGCTGAAGAA GCCCCTTATG ATGCCATTCA TGTGGGAGCT 480
GCAGCCCCTG TTGTACCCCA GGCGCTAATA GATCAGTTAA AGCCCGGAGG AAGATTGATA 540
TTGCCTGTTG GTCCTGCAGG CGGAAACCAA ATGTTGGAGC AGTATGACAA GCTACAAGAT 600
GGCAGCATCA AAATGMRNCC TCTGATGGGG GTGATATACG TGCCTTTAAC AGATAAAGAA 660
AAGCAGTGGT CCAGGGATGA ATTG 684

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 226 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 22...22
(D) OTHER INFORMATION: Ile or Leu

(A) NAME/KEY:Other
(B) LOCATION: 119...119
(D) OTHER INFORMATION: Ile or Val

(A) NAME/KEY:Other
(B) LOCATION: 205...205

(D) OTHER INFORMATION: Lys or Arg

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Ala Trp Lys Ser Gly Gly Ala Ser His Ser Glu Leu Ile His Asn Leu
 1               5                  10                  15
Arg Lys Asn Gly Ile Xaa Lys Thr Asp Lys Val Phe Glu Val Met Leu
            20                  25                  30
Ala Thr Asp Arg Ser His Tyr Ala Lys Cys Asn Pro Tyr Met Asp Ser
        35                  40                  45
Pro Gln Ser Ile Gly Phe Gln Ala Thr Ile Ser Ala Pro His Met His
    50                  55                  60
Ala Tyr Ala Leu Glu Leu Leu Phe Asp Gln Leu His Glu Gly Ala Lys
65                  70                  75                  80
Ala Leu Asp Val Gly Ser Gly Ser Gly Ile Leu Thr Ala Cys Phe Ala
                85                  90                  95
Arg Met Val Gly Cys Thr Gly Lys Val Ile Gly Ile Asp His Ile Lys
            100                 105                 110
Glu Leu Val Asp Asp Ser Xaa Asn Asn Val Arg Lys Asp Asp Pro Thr
        115                 120                 125
Leu Leu Ser Ser Gly Arg Val Gln Leu Val Val Gly Asp Gly Arg Met
    130                 135                 140
Gly Tyr Ala Glu Glu Ala Pro Tyr Asp Ala Ile His Val Gly Ala Ala
145                 150                 155                 160
Ala Pro Val Val Pro Gln Ala Leu Ile Asp Gln Leu Lys Pro Gly Gly
                165                 170                 175
Arg Leu Ile Leu Pro Val Gly Pro Ala Gly Gly Asn Gln Met Leu Glu
            180                 185                 190
Gln Tyr Asp Lys Leu Gln Asp Gly Ser Ile Lys Met Xaa Pro Leu Met
        195                 200                 205
Gly Val Ile Tyr Val Pro Leu Thr Asp Lys Glu Lys Gln Trp Ser Arg
    210                 215                 220
Trp Lys
225
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 227 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 22...22
(D) OTHER INFORMATION: Ile or Leu

(A) NAME/KEY:Other
(B) LOCATION: 119...119
(D) OTHER INFORMATION: Ile or Val

(A) NAME/KEY:Other
(B) LOCATION: 205...205
(D) OTHER INFORMATION: Lys or Arg

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Ala Trp Lys Ser Gly Gly Ala Ser His Ser Glu Leu Ile His Asn Leu
 1               5                  10                  15
Arg Lys Asn Gly Ile Xaa Lys Thr Asp Lys Val Phe Glu Val Met Leu
            20                  25                  30
Ala Thr Asp Arg Ser His Tyr Ala Lys Cys Asn Pro Tyr Met Asp Ser
        35                  40                  45
Pro Gln Ser Ile Gly Phe Gln Ala Thr Ile Ser Ala Pro His Met His
    50                  55                  60
Ala Tyr Ala Leu Glu Leu Leu Phe Asp Gln Leu His Glu Gly Ala Lys
65                  70                  75                  80
Ala Leu Asp Val Gly Ser Gly Ser Gly Ile Leu Thr Ala Cys Phe Ala
                85                  90                  95
Arg Met Val Gly Cys Thr Gly Lys Val Ile Gly Ile Asp His Ile Lys
            100                 105                 110
Glu Leu Val Asp Asp Ser Xaa Asn Asn Val Arg Lys Asp Asp Pro Thr
        115                 120                 125
Leu Leu Ser Ser Gly Arg Val Gln Leu Val Val Gly Asp Gly Arg Met
    130                 135                 140
Gly Tyr Ala Glu Glu Ala Pro Tyr Asp Ala Ile His Val Gly Ala Ala
145                 150                 155                 160
Ala Pro Val Val Pro Gln Ala Leu Ile Asp Gln Leu Lys Pro Gly Gly
                165                 170                 175
Arg Leu Ile Leu Pro Val Gly Pro Ala Gly Gly Asn Gln Met Leu Glu
            180                 185                 190
Gln Tyr Asp Lys Leu Gln Asp Gly Ser Ile Lys Met Xaa Pro Leu Met
        195                 200                 205
Gly Val Ile Tyr Val Pro Leu Thr Asp Lys Glu Lys Gln Trp Ser Arg
    210                 215                 220
Asp Glu Leu
225
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 944 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
CGTCCCGCTT CCTGTTGCCC TCCACGGCCG CCCGCCGCCG GTTCCTCCAC CACCTCCTCG   60
CCGCGCCCGC GCCCCCGAGG CCGCCGCAGC TTCGGCGCTG CTCCCCGTAC CACTGGATGG  120
CGCAATTTTG GGCTGAAGGA TCACTGGAGA AGAACAACGC TCTGGTTGAA TACCTGAAAC  180
AGTATGGTGT TGTTCGAACC GATAAAGTGG CAGAAGTTAT GGAAACTATC GACCGAGCCT  240
TATTTGTACC GGAGGGCTTT ACCCCTTACA CCGACAGTCC TATGCCTATT GGTTACAATG  300
CAACAATATC TGCTCCTCAC ATGCACGCAA CCTGCTTAGA ACTGTTGAAG GATTATTTAC  360
AGCCAGGCAT GCATGCTCTG GACGTTGGAT CAGGCAGTGG TTACTTGACT GCTTGCTTTG  420
CAATGATGGT CGGACCAGAA GGTCGCGCAG TGGGGATTGA ACACATTCCT GAACTCGTTG  480
TTGCTTCTAC TGAAAATGTC GAACGGAGTG CTGCAGCAGC ACTAATGAAG GATGGTTCAC  540
TTTCTTTTCA TGTTTCAGAT GGAAGGCTTG GCTGGCCGGA TGCGGCGCCA TACGATGCTA  600
TTCATGTGGG CGCAGCGGCA CCTGAGATCC CTCGGCCACT GCTGGAGCAG CTGAAGCCTG  660
GCGGGCGGAT GGTCATACCC GTTGGCACAT ACTCTCAGGA CCTGCAGGTG ATTGACAAGA  720
GCGCCGACGG ATCCACCAGC GTCCGCAACG ATGCCTCTGT TCGCTACGTC CCTCTGACCA  780
GCCGCTCTGC TCAGCTGCAG GACAGCTGAG ATCCTTCGCT CTGGATCTGG AAATGTGTGT  840
GTATATATGT GAGTGCCGAT GATCTTTGTC TACCAATGTG GCGTCTGATG TTTTAGATGG  900
TTTGGTTTTG TATAATGCTT ACTGCTGGTT GATGTTGCTT AAAA                    944
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 230 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Ala Gln Phe Trp Ala Glu Gly Ser Leu Glu Lys Asn Asn Ala Leu
 1               5                  10                  15
Val Glu Tyr Leu Lys Gln Tyr Gly Val Val Arg Thr Asp Lys Val Ala
            20                  25                  30
Glu Val Met Glu Thr Ile Asp Arg Ala Leu Phe Val Pro Glu Gly Phe
        35                  40                  45
Thr Pro Tyr Thr Asp Ser Pro Met Pro Ile Gly Tyr Asn Ala Thr Ile
    50                  55                  60
Ser Ala Pro His Met His Ala Thr Cys Leu Glu Leu Leu Lys Asp Tyr
65                  70                  75                  80
Leu Gln Pro Gly Met His Ala Leu Asp Val Gly Ser Gly Ser Gly Tyr
                85                  90                  95
Leu Thr Ala Cys Phe Ala Met Met Val Gly Pro Glu Gly Arg Ala Val
            100                 105                 110
Gly Ile Glu His Ile Pro Glu Leu Val Val Ala Ser Thr Glu Asn Val
        115                 120                 125
Glu Arg Ser Ala Ala Ala Ala Leu Met Lys Asp Gly Ser Leu Ser Phe
    130                 135                 140
His Val Ser Asp Gly Arg Leu Gly Trp Pro Asp Ala Ala Pro Tyr Asp
145                 150                 155                 160
Ala Ile His Val Gly Ala Ala Ala Pro Glu Ile Pro Arg Pro Leu Leu
                165                 170                 175
Glu Gln Leu Lys Pro Gly Gly Arg Met Val Ile Pro Val Gly Thr Tyr
            180                 185                 190
Ser Gln Asp Leu Gln Val Ile Asp Lys Ser Ala Asp Gly Ser Thr Ser
        195                 200                 205
Val Arg Asn Asp Ala Ser Val Arg Tyr Val Pro Leu Thr Ser Arg Ser
    210                 215                 220
Ala Gln Leu Gln Asp Ser
225                 230
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 231 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 18...18

(D) OTHER INFORMATION: Asp or Glu

(A) NAME/KEY:Other
(B) LOCATION: 41...41
(D) OTHER INFORMATION: Asn or Ala

(A) NAME/KEY:Other
(B) LOCATION:52...52
(D) OTHER INFORMATION: Ile or Thr

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Ala Gln Phe Trp Ala Glu Gly Ser Leu Glu Lys Asn Asn Ala Leu
 1               5                  10                  15
Val Xaa Tyr Leu Lys Gln Tyr Gly Val Val Arg Thr Asp Lys Val Ala
            20                  25                  30
Glu Val Met Glu Thr Ile Asp Arg Xaa Leu Phe Val Pro Glu Gly Phe
        35                  40                  45
Thr Pro Tyr Xaa Asp Xaa Pro Met Pro Ile Gly Tyr Asn Ala Thr Ile
    50                  55                  60
Ser Ala Pro His Met His Ala Thr Cys Leu Glu Leu Leu Lys Asp Tyr
65                  70                  75                  80
Leu Gln Pro Gly Met His Ala Leu Asp Val Gly Ser Gly Ser Gly Tyr
                85                  90                  95
Leu Thr Ala Cys Phe Ala Met Met Val Gly Pro Glu Gly Arg Ala Val
            100                 105                 110
Gly Ile Glu His Ile Pro Glu Leu Val Xaa Ala Ser Thr Glu Asn Val
        115                 120                 125
Glu Arg Ser Ala Ala Ala Ala Leu Met Lys Asp Gly Ser Leu Xaa Phe
    130                 135                 140
His Val Xaa Asp Gly Arg Leu Gly Trp Pro Asp Xaa Ala Pro Tyr Asp
145                 150                 155                 160
Ala Ile His Val Gly Ala Ala Ala Pro Glu Ile Pro Arg Pro Leu Leu
                165                 170                 175
Glu Gln Leu Lys Pro Gly Gly Arg Met Val Ile Pro Val Gly Thr Tyr
            180                 185                 190
Ser Gln Asp Leu Gln Val Ile Asp Lys Ser Xaa Asp Gly Ser Thr Xaa
        195                 200                 205
Val Xaa Asn Asp Ala Xaa Val Arg Tyr Val Pro Leu Thr Ser Arg Ser
    210                 215                 220
Ala Gln Leu Gln Asp Ser
225                 230
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
CTCGAGTCTA GAGGATCCTT TGTTTAACTT TAAGAAGGAA AGCTAGCCAT GGCCTGGAAA  60
TCCGGCGG                                                           68
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1218 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 85...765
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
GTAATACGAC TCACTATAGG GCGAATTGGG TACCTCGAGT CTAGAGGATC CTTTGTTTAA  60
CTTTAAGAAG GAAAGCTAGC CATG GCC TGG AAA TCC GGC GGC GCC AGC CAC     111
                       Ala Trp Lys Ser Gly Gly Ala Ser His
                        1               5

TCG GAG CTA ATC CAC AAT CTC CGC AAA AAT GGA ATC ATC AAG ACA GAT   159
Ser Glu Leu Ile His Asn Leu Arg Lys Asn Gly Ile Ile Lys Thr Asp
10                  15                  20                  25

AAA GTA TTT GAA GTG ATG CTG GCT ACA GAC CGC TCC CAC TAT GCA AAA   207
Lys Val Phe Glu Val Met Leu Ala Thr Asp Arg Ser His Tyr Ala Lys
                30                  35                  40

TGT AAC CCA TAC ATG GAT TCT CCA CAA TCA ATA GGT TTC CAA GCA ACA   255
Cys Asn Pro Tyr Met Asp Ser Pro Gln Ser Ile Gly Phe Gln Ala Thr
            45                  50                  55

ATC AGT GCT CCA CAC ATG CAT GCA TAT GCG CTA GAA CTT CTA TTT GAT   303
Ile Ser Ala Pro His Met His Ala Tyr Ala Leu Glu Leu Leu Phe Asp
        60                  65                  70

CAG TTG CAT GAA GGA GCT AAA GCT CTT GAT GTA GGA TCT GGA AGT GGA   351
Gln Leu His Glu Gly Ala Lys Ala Leu Asp Val Gly Ser Gly Ser Gly
    75                  80                  85

ATC CTT ACT GCA TGT TTT GCA CGT ATG GTT GGA TGT ACT GGA AAA GTC   399
Ile Leu Thr Ala Cys Phe Ala Arg Met Val Gly Cys Thr Gly Lys Val
90                  95                  100                 105

ATA GGA ATT GAT CAC ATT AAA GAG CTA GTA GAT GAC TCA GTA AAT AAT   447
Ile Gly Ile Asp His Ile Lys Glu Leu Val Asp Asp Ser Val Asn Asn
                110                 115                 120

GTC AGG AAG GAC GAT CCA ACA CTT CTG TCT TCA GGG AGA GTA CAG CTT   495
Val Arg Lys Asp Asp Pro Thr Leu Leu Ser Ser Gly Arg Val Gln Leu
            125                 130                 135

GTT GTG GGG GAT GGA AGA ATG GGA TAT GCT GAA GAA GCC CCT TAT GAT   543
Val Val Gly Asp Gly Arg Met Gly Tyr Ala Glu Glu Ala Pro Tyr Asp
        140                 145                 150

GCC ATT CAT GTG GGA GCT GCA GCC CCT GTT GTA CCC CAG GCG CTA ATA   591
Ala Ile His Val Gly Ala Ala Ala Pro Val Val Pro Gln Ala Leu Ile
    155                 160                 165
```

```
GAT CAG TTA AAG CCC GGA GGA AGA TTG ATA TTG CCT GTT GGT CCT GCA     639
Asp Gln Leu Lys Pro Gly Gly Arg Leu Ile Leu Pro Val Gly Pro Ala
170                 175                 180                 185

GGC GGA AAC CAA ATG TTG GAG CAG TAT GAC AAG CTA CAA GAT GGC AGC     687
Gly Gly Asn Gln Met Leu Glu Gln Tyr Asp Lys Leu Gln Asp Gly Ser
                190                 195                 200

ATC AAA ATG AAG CCT CTG ATG GGG GTG ATA TAC GTG CCT TTA ACA GAT     735
Ile Lys Met Lys Pro Leu Met Gly Val Ile Tyr Val Pro Leu Thr Asp
            205                 210                 215

AAA GAA AAG CAG TGG TCC AGG GAT GAA TTG TAAAAGCAAC ATCAGCTTGA CCAG  789
Lys Glu Lys Gln Trp Ser Arg Asp Glu Leu
        220                 225

TATAAAATTA CAGTGGATTG CTCATCTCAG TCCTCAAAGC TTTTTGAAAA CCAACACCAT  849
CACAGCTTGT TTTGGACTTT GTTACACTGT TATTTTCAGC ATGAAAATGT GTGTTTTTTT  909
AGGGTTTCTG ATTCTTCAAA GAGGCACAGA GCCAAATTGG TAGAGGAAGG ATGCAAAGTA  969
TAAATTTGTG TAATATTACT TTAACATGCC CATATTTTAC TTGGAAATAT TAAAAGAAAG 1029
GGTTCTGTAA AATGGAAAAC TTAGTTTGTG AATTGATTTT GAGGAGTGGT TTTTCTTTTC 1089
TTGGACACTT AATTCTGTTC TGATATTAAT TTATCAGATT GCTTTTGTGC ATTGGATAAC 1149
ACCACCATTC ACAAGTTAAG ATTCTTGGTA TTTGGATATC TGTTAGATGC TACTAAAAAA 1209
AAGGAATTC                                                         1218
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 324 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATAAATAATG TCAGGAAGGA CGATCCAACA CTTCTGTCTT CAGGGAGAGT ACAGCTTGTT  60
GTGGGGGATG GAAGAATGGG ATATGCTGAA GAAGCCCCTT ATGATGCCAT TCATGTGGGA 120
GCTGCAGCCC CTGTTGTACC CCAGGCGCTA ATAGATCAGT TAAAGCCCGG AGGAAGATTG 180
ATATTGCCTG TTGGTCCTGC AGGCGGAAAC CAAATGTTGG AGCAGTATGA CAAGCTACAA 240
GATGGCAGCA TCAAAATGAG GCCTCTGATG GGGGTGATAT ACGTGCCTTT AACAGATAAA 300
GAAAAGCAGT GGTCCAGGTG GAAG                                        324
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAATACGACT CACTATAGGG 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TCTGGRATRT GYTCRATNCC CAT 23

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCGAATTCT AYYTNAARCA RTAYGGNGT 29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCAAAGCTTT TRTCDATNAC YTGNAG 26

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 4...4
(D) OTHER INFORMATION: L-isoaspartyl

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Val Tyr Pro Xaa His Ala
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 5...5
(D) OTHER INFORMATION: L-isoaspartyl

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Lys Ala Ser Ala Xaa Leu Ala Lys Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 3...3
(D) OTHER INFORMATION: L-isoaspartyl

(A) NAME/KEY:Modified Base
(B) LOCATION: 4...4
(D) OTHER INFORMATION: Phe-NH2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ala Ala Xaa Xaa
1

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 4...4
(D) OTHER INFORMATION: D-aspartyl

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Val Tyr Gly Xaa Pro Ala
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acids
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 5...5
(D) OTHER INFORMATION: D-aspartyl

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Lys Ala Ser Ala Xaa Leu Ala Lys Tyr
1 5

WHAT WE CLAIMED IS:

1. An isolated recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase obtained by expression of a polynucleotide having a sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

2. An isolated recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase having an amino acid sequence selected from the group consisting of SEQ NO:3 and SEQ ID NO:4.

3. An isolated polynucleotide having the coding sequence of the sequence indicated as SEQ ID NO:5, said polynucleotide coding for a plant L-isoaspartyl protein methyltransferase.

4. An isolated recombinant plant L-isoaspartyl protein methyltransferase obtained by expression of SEQ ID NO:5.

5. A purified plant L-isoaspartyl protein methyltransferase having the amino acid sequence SEQ ID NO:7, obtained from wheat germ.

6. A method of recombinantly producing human L-isoaspartyl/D-aspartyl protein methyltransferase, comprising:

modifying a plasmid that contains the T7 promoter region and the full coding region of human L-isoaspartyl/D-aspartyl protein methyltransferase, using oligonucleotides, to provide multiple cloning sites, an efficient ribosome binding site, and a strong translational initiator region, said initiator region being designed to function in bacterial and/or eukaryotic expression system;

transfecting the constructed vector into a host that contains an inducible T7 polymerase gene; and

inducing overexpression of the methyltransferase, whereby the methyltransferase is produced.

7. The method of Claim 6, wherein the inducible T7 polymerase gene is induced by heat shock or inclusion of a chamical and the inducing step comprises addition of IPTG.

8. The method of Claim 7, wherein the said chamical inducing the T7 polymerase gene is isopropyl β-D-thiogalactopyranoside (IPTG)

9. The method of recombinantly producing human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 6, wherein said full coding region is that of isozyme II of human L-isoaspartyl/D-aspartyl protein methyltransferase having the sequence indicated as SEQ ID NO:2.

10. The method of recombinantly producing human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 9, wherein said plasmid to be modified is obtained from a commercial cDNA library derived from human brain tissue using a radiolabeled 769 bp *HaeIII* fragment from the coding region of a 1580 bp murine methyltransferase cDNA.

11. The method of recombinantly producing human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 10, wherein the plasmid to be modified is plasmid pDM2 having the sequence indicated as SEQ ID NO:9.

12. The method of recombinantly producing human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 6, wherein said modification is conducted by replacing the region between the T7-promoter site and the start codon of the enzyme, which is 107 bp from the KpnI site to the NarI site, with a synthetic fragment containing ribosome binding and initiator sites, said fragment having the sequence indicated as SEQ ID NO:8.

13. The method of recombinantly producing human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 6, wherein said host is E. coli strain BL21(DE3) or any other suitable strain.

14. The method of recombinantly producing human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 6, wherein the concentration of said IPTG is 0.03 mM to 8 mM.

15. A method of purifying recombinantly produced human L-isoaspartyl/D-aspartyl protein methyltransferase present in a lysed bacterial extract in which methyltransferase expression has occurred, comprising:

adding a nucleotide precipitant to the extract to remove DNA present in the extract subsequent to removing the cellular debris therefrom;

precipitating the methyltransferase with ammonium sulfate;

removing the ammonium sulfate by dialysis; and

purifying the methyltransferase from the dialysate by using a DEAE-cellulose anion-exchange chromatography column.

16. The method of purifying recombinantly produced human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 15, wherein said nucleotide precipitant is protamine sulfate or polyethyleneimine.

17. The method of purifying recombinantly produced human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 15, wherein the addition of said protamine sulfate is 0.1 volumes of a 4% solution thereof.

18. The method of purifying recombinantly produced human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 15, wherein the saturation of said ammonium sulfate is 50% to 60%.

19. The method of purifying recombinantly produced human L-isoaspartyl/D-aspartyl protein methyltransferase according to Claim 15, wherein said column is fully equilibrated before the loading of the dialysate.

20. A method of purifying plant L-isoaspartyl protein methyltransferase from wheat, comprising:

obtaining a crude cytosol of raw wheat;

fractionating the crude cytosol by DEAE-cellulose chromatography;

adding ammonium sulfate to the pooled active fractions in the presence of a protein carrier;

fractionating the resulting material by reverse ammonium sulfate gradient solubilization; and

purifying the pooled active fractions by gel filtration chromatography, whereby the methyltransferase is purified as a monomeric 28,000 Da species.

21. The method of purifying plant L-isoaspartyl protein methyltransferase according to Claim 20, wherein said crude cytosol is obtained from wheat germ.

22. The method of purifying plant L-isoaspartyl protein methyltransferase according to Claim 20, wherein said fractionation by the DEAE-cellulose chromatography is conducted at pH 9.3.

23. The method of purifying plant L-isoaspartyl protein methyltransferase according to Claim 20, wherein said ammonium sulfate is added to 80% saturation.

24. The method of purifying plant L-isoaspartyl protein methyltransferase according to Claim 20, wherein said protein carrier is Celite 545.

25. The method of purifying plant L-isoaspartyl protein methyltransferase according to Claim 20, wherein said reverse ammonium sulfate gradient solubilization is conducted with a linear gradient decreasing from 80% to 0% saturation in ammonium sulfate.

26. The method of purifying plant L-isoaspartyl protein methyltransferase according to Claim 20, wherein said pooled active fractions after the fractionation by reverse ammonium sulfate gradient solubilization contains 26-31% saturated ammonium sulfate.

27. The method of purifying plant L-isoaspartyl protein methyltransferase according to Claim 20, wherein said gel filtration chromatography is Sephacryl S-200 gel filtration chromatography.

28. The method of purifying plant L-isoaspartyl protein methyltransferase according to Claim 20, further comprising, subsequent to the gel filtration chromatography, dialyzing the purified methyltransferase and introducing the dialyzed methyltransferase to a Mono Q anion exchange column.

29. An expression vector for human L-isoaspartyl/D-aspartyl protein methyltransferase constructed by modifying a plasmid that contains the full coding region of the human L-isoaspartyl/D-aspartyl protein methyltransferase, using oligonucleotides, to provide multiple cloning sites, an efficient ribosome-binding site, and a strong translational initiator region, said initiator region being designed to function in a bacterial and/or eukaryotic expression system.

30. The expression vector according to Claim 29, wherein said full coding region is that of isozyme II of human L-isoaspartyl/D-aspartyl protein methyltransferase having the sequence indicated as SEQ ID NO:2.

31. The expression vector according to Claim 30, wherein the plasmid to be modified is plasmid pDM2.

32. The expression vector according to Claim 29, wherein said modification is conducted by replacing the region between the T7-promoter site and the start codon of the enzyme, which is 107 bp from the KpnI site to the NarI site, with a synthetic fragment containing ribosome binding and initiator sites, said fragment having the sequence indicated as SEQ ID NO:8.

33. A method of treatment for a medical condition associated with an increase in L-isoaspartyl/D-aspartyl residues of polypeptides in a tissue, comprising:

administering to the tissue an amount of methyltransferase sufficient to convert said L-isoaspartyl/D-aspartyl residues to L-aspartyl residues in the tissue.

34. The method of treatment for a condition according to Claim 33, wherein said condition is crosslinking of matrix proteins and degradation of flexibility of tissues.

35. The method of treatment for a condition according to Claim 33, wherein said condition is cataracts.

36. The method of treatment for a condition according to Claim 33, wherein said condition is degradation of corneal flexibility.

37. The method of treatment for a condition according to Claim 33, wherein said condition is formation of plaque in brain tissues.

38. The method of treatment for a condition according to Claim 33, wherein said condition is degradation of cellular function in brain tissues.

39. The method of treatment for a condition according to Claim 33, wherein said condition is degradation of flexibility in a vascular system.

40. The method of treatment for a condition according to Claim 33, wherein said condition is infertility related to eggs and/or sperm.

41. The method of treatment for a condition according to Claim 33, wherein said condition is formation of fibrosis in tissues.

42. The method of treatment for a condition according to Claims 33, wherein said methyltransferase is an isolated recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase

43. The method of treatment for a condition according to Claim 33, wherein said methyltransferase is administered in conjunction with S-adenosylmethionine.

44. The method of treatment for a condition according to Claim 33, wherein said methyltransferase is a purified plant L-isoaspartyl protein methyltransferase.

45. A method of diagnosis of a state where L-isoaspartyl/D-aspartyl residues are accumulated in a patient, comprising:

obtaining a biological sample containing protein from said patient; and

measuring the content of L-isoaspartyl/D-aspartyl residues accumulated in said protein, by using methyltransferase as a probe.

46. A pharmaceutical preparation for treatment of a condition associated with an increase in L-isoaspartyl/D-aspartyl residues of polypeptides in a tissue, comprising:

human L-isoaspartyl/D-aspartyl protein methyltransferase; and

a pharmaceutically acceptable carrier.

47. The pharmaceutical preparation for treatment of a condition according to Claim 46, wherein said preparation is a skin or hair cream.

48. The pharmaceutical preparation for treatment of a condition according to Claim 46, wherein said preparation is eye drops.

49. The pharmaceutical preparation for treatment of a condition according to Claim 46, wherein said preparation is an injectable solution.

50. The pharmaceutical preparation for treatment of a condition according to Claim 46, wherein said preparation contains S-adenosylmethionine as a substrate of the enzyme.

51. A method of treatment for plant protein degradation associated with an increase in L-isoaspartyl residues of polypeptides in a tissue, comprising:

administering to the tissue an amount of plant L-isoaspartyl protein methyltransferase sufficient to convert said L-isoaspartyl residues to L-aspartyl residues in the tissue.

52. The method of treatment for plant protein degradation according to Claim 51, wherein said plant protein degradation is caused by desiccation, aging, and environmental stress.

53. The method of treatment for plant protein degradation according to Claim 51, wherein said treatment is applied to seed tissues and/or seedling tissues.

54. The method of treatment for plant protein degradation according to Claim 51, wherein said methyltransferase is administered in conjunction with S-adenosylmethionine.

55. A method of recombinantly producing plant L-isoaspartyl protein methyltransferase, comprising:

modifying a plasmid that contains the T7 promoter region and the full coding region of plant L-isoaspartyl protein methyltransferase, using oligonucleotides, to provide multiple cloning sites, an efficient

ribosome binding site, and a strong translational initiator region, said initiator region being designed to function in bacterial and/or eukaryotic expression system;

transfecting the constructed vector into a host that contains an inducible T7 polymerase gene; and

inducing overexpression of the methyltransferase, whereby the methyltransferase is produced.

56. A method of purifying recombinantly produced plant L-isoaspartyl protein methyltransferase present in a lysed bacterial extract in which methyltransferase expression has occurred, comprising:

adding a nucleotide precipitant to the extract to remove DNA present in the extract subsequent to removing the cellular debris therefrom;

precipitating the methyltransferase with ammonium sulfate;

removing the ammonium sulfate by dialysis; and

purifying the methyltransferase from the dialysate by using a DEAE-cellulose anion-exchange chromatography column.

57. The method of Claim 56, wherein the nucleotide precipitant comprises protamine sulfate or polyethyleneimine.

58. A methyltransferase for use as a medicament in the treatment of a medical condition associated with an increase in L-isoaspartyl/D-aspartyl residues of polypeptides in a tissue.

59. The methyltransferase of Claim 58, wherein, said condition is crosslinking of matrix proteins and degradation of flexibility of tissues.

60. The methyltransferase of Claim 58, wherein said condition is cataracts, degradation of corneal flexibility, formation of plaque in brain tissues, degradation of cellular function in brain tissues, degradation of flexibility in a vascular system, infertility related to eggs and/or sperm, or formation of fibrosis in tissues.

61. The methyltransferase of Claim 58, wherein said methyltransferase is an isolated recombinant human L-isoaspartyl/D-aspartyl protein methyltransferase.

62. The methyltransferase of Claim 58, in combination with S-adenosylmethionine.

63. The methyltransferase of Claim 58, wherein said methyltransferase is a purified plant L-isoaspartyl protein methyltransferase.

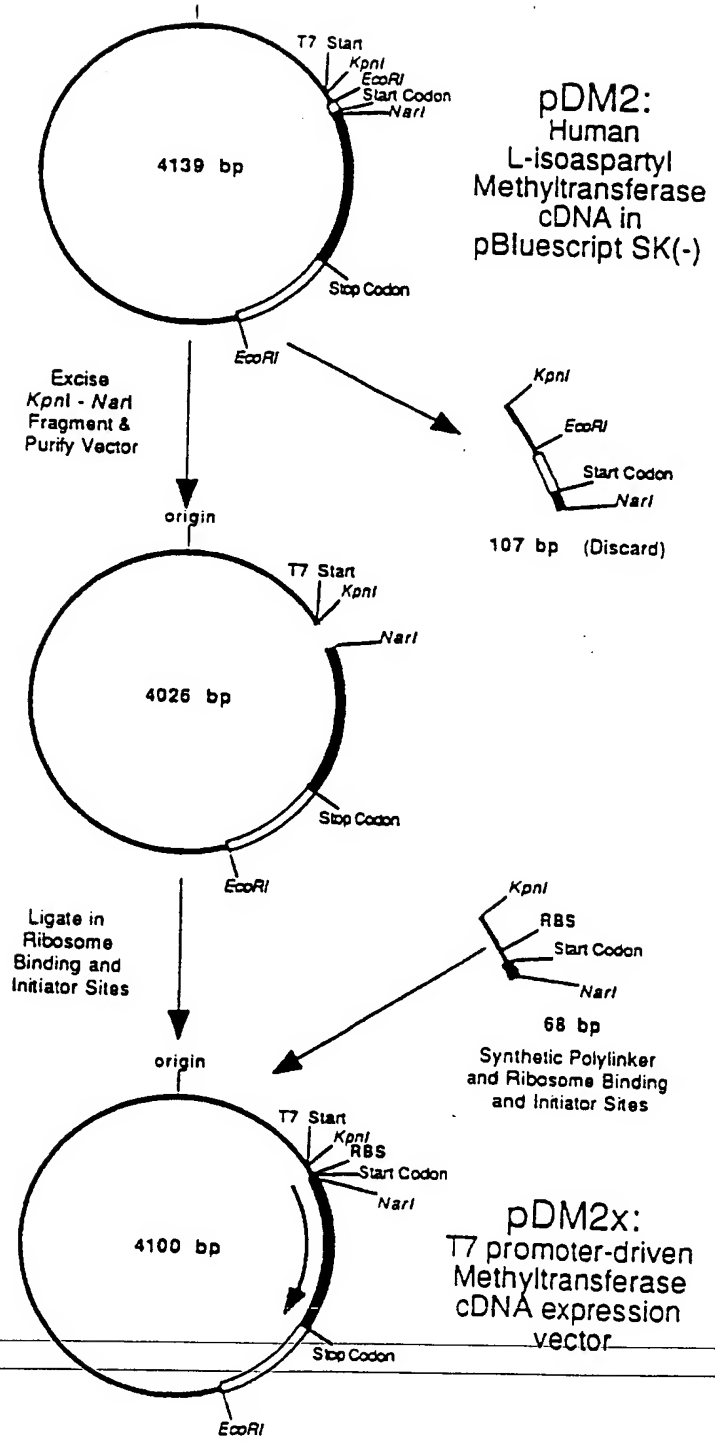
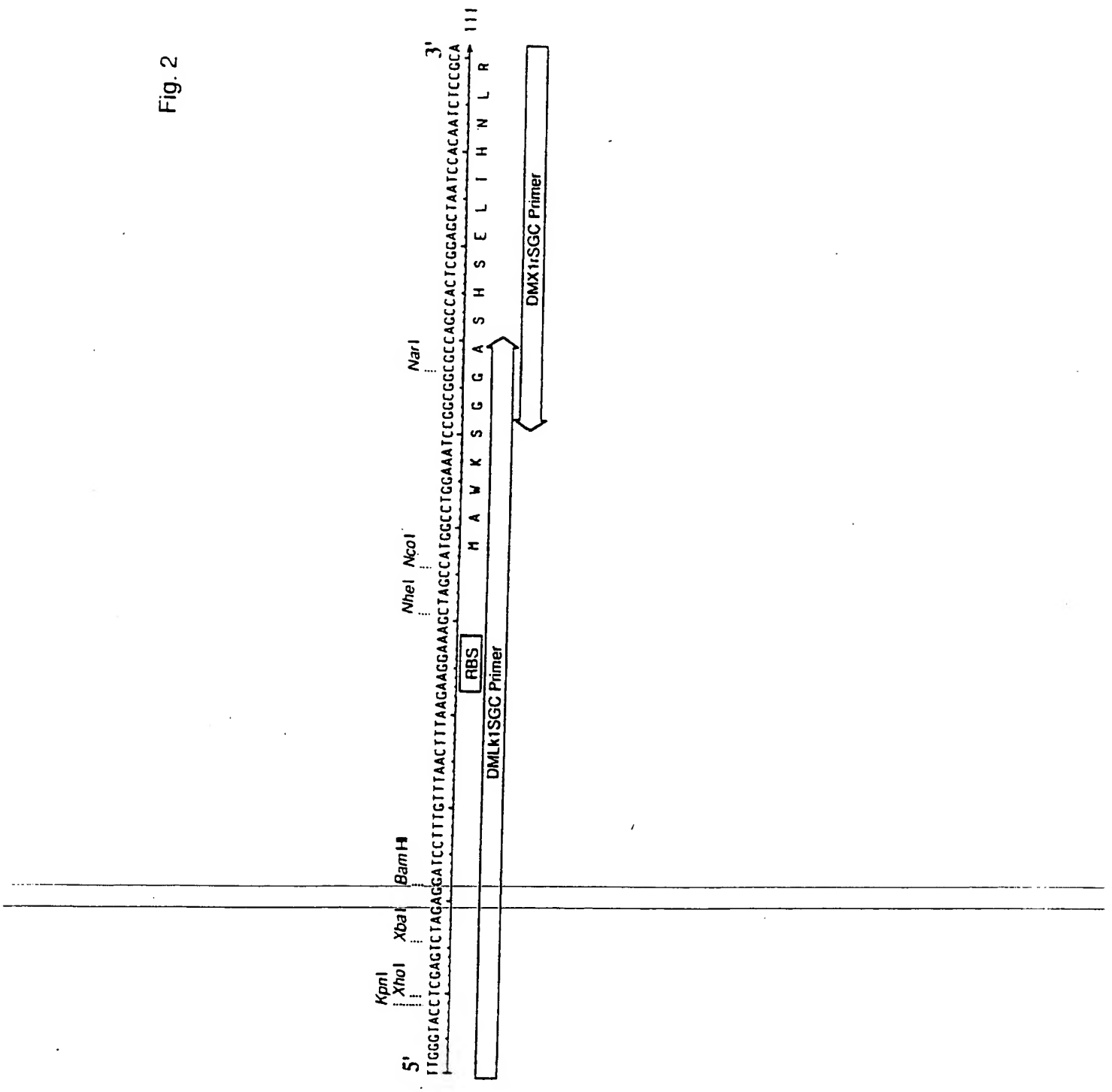

T7 Start
KpnI
EcoRI
Start Codon
NarI
4139 bp
Stop Codon
EcoRI
pDM2:
Human
L-isoaspartyl
Methyltransferase
cDNA in
pBluescript SK(-)
Excise
KpnI - NarI
Fragment &
Purify Vector
KpnI
EcoRI
Start Codon
NarI
107 bp (Discard)
origin
T7 Start
KpnI
NarI
4026 bp
Stop Codon
EcoRI
Ligate in
Ribosome
Binding and
Initiator Sites
KpnI
RBS
Start Codon
NarI
68 bp
Synthetic Polylinker
and Ribosome Binding
and Initiator Sites
origin
T7 Start
KpnI
RBS
Start Codon
NarI
4100 bp
Stop Codon
EcoRI
pDM2x:
T7 promoter-driven
Methyltransferase
cDNA expression
vector

Fig. 2


5'
3'
KpnI
XhoI
XbaI
BamHI
NheI
NcoI
NarI
RBS
DMLk1SGC Primer
DMX1rSGC Primer

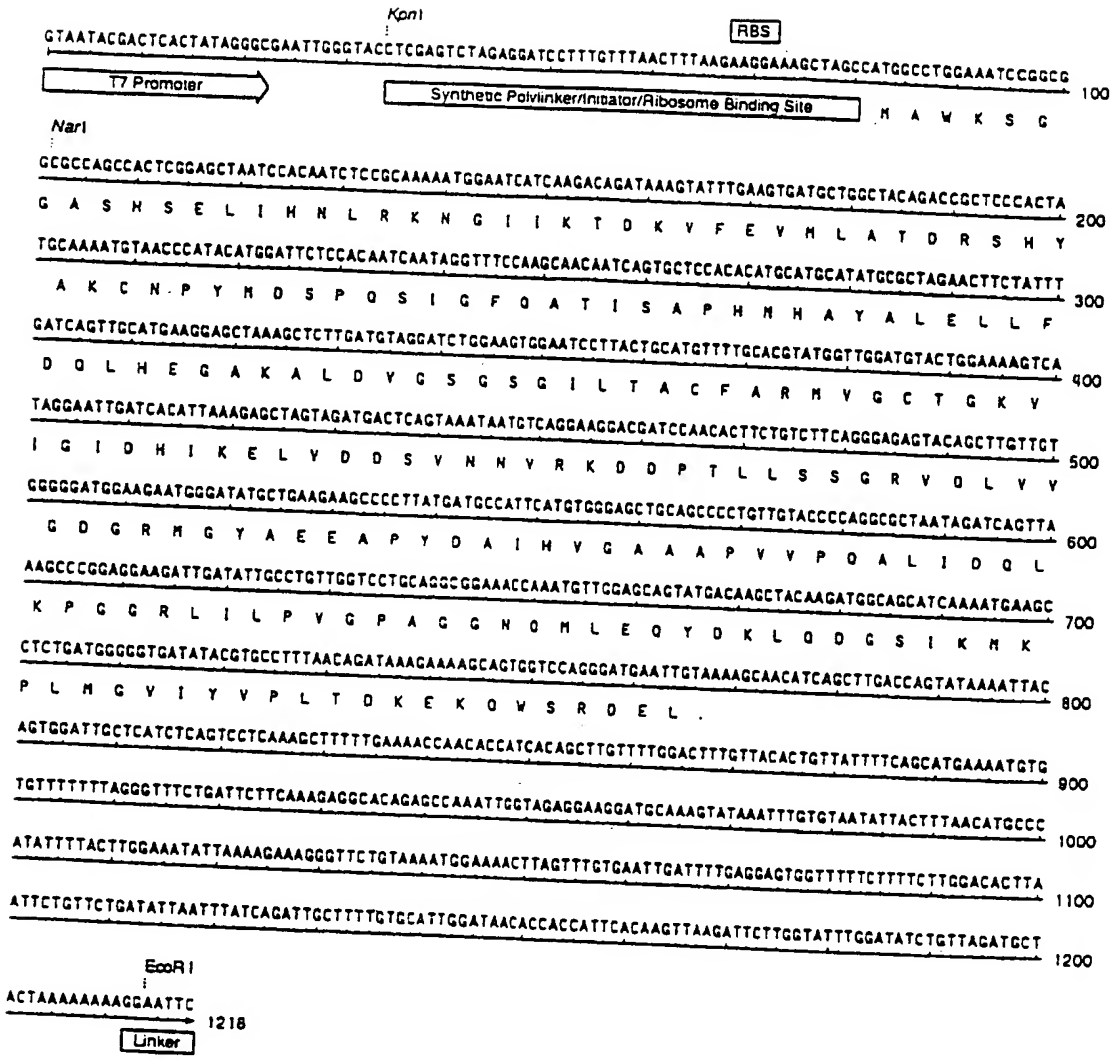

KpnI
RBS
T7 Promoter
Synthetic Polylinker/Initiator/Ribosome Binding Site
NarI
EcoRI
Linker
100
200
300
400
500
600
700
800
900
1000
1100
1200
1218

Fig. 4

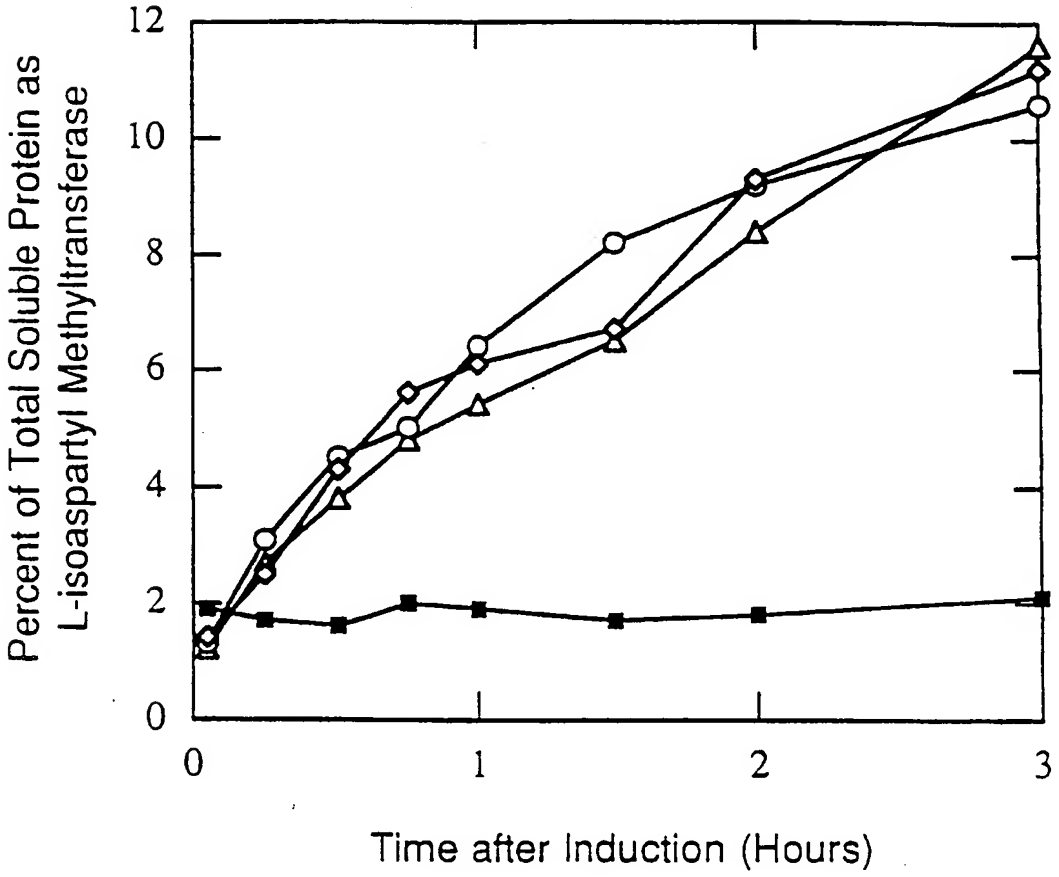

Percent of Total Soluble Protein as L-isoaspartyl Methyltransferase
12
10
8
6
4
2
0
0
1
2
3
Time after Induction (Hours)

Fig. 5

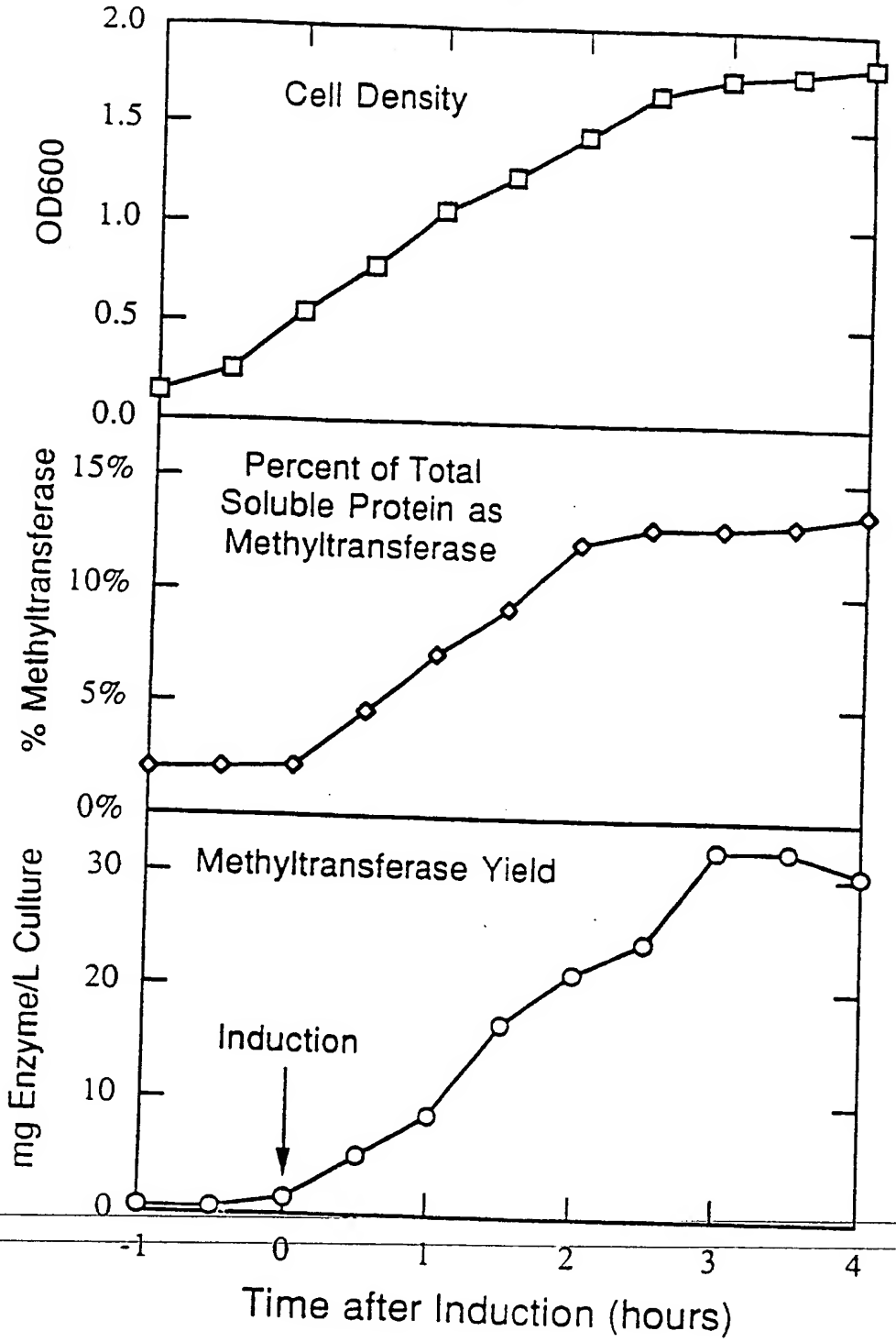

2.0
1.5
1.0
0.5
0.0
OD600
Cell Density
15%
10%
5%
0%
% Methyltransferase
Percent of Total
Soluble Protein as
Methyltransferase
30
20
10
0
mg Enzyme/L Culture
Methyltransferase Yield
Induction
-1
0
1
2
3
4
Time after Induction (hours)

Fig. 6

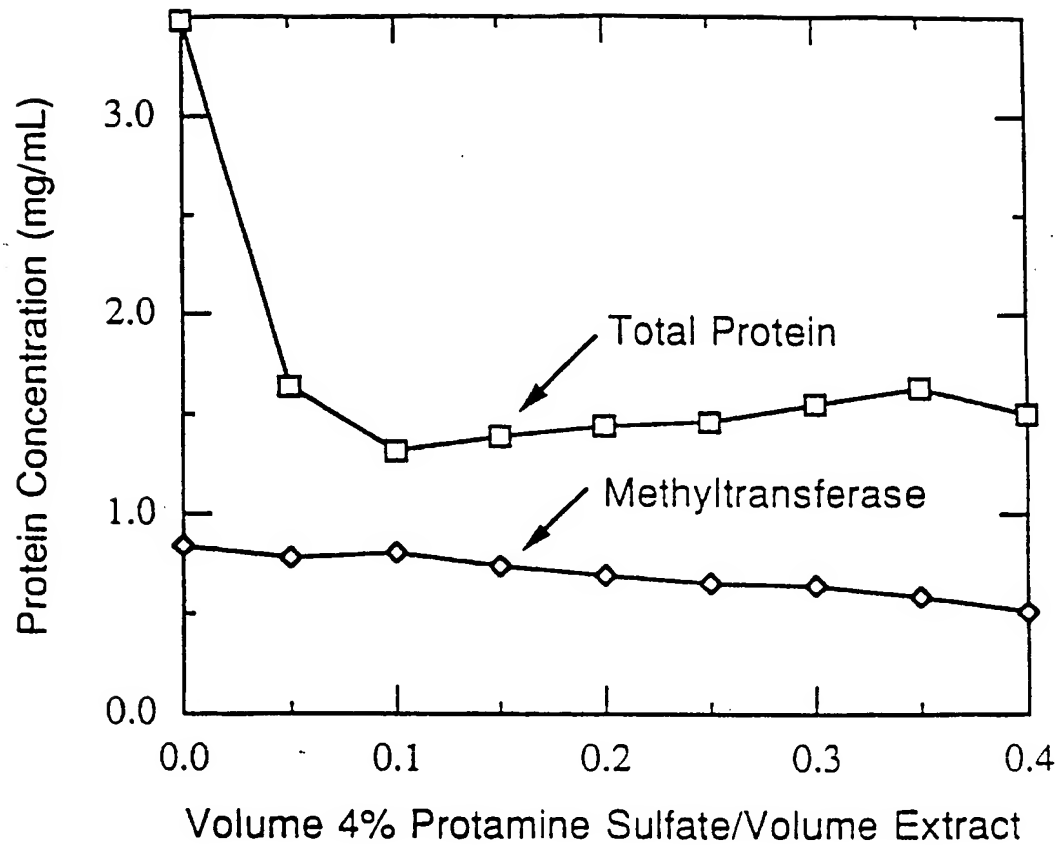

Protein Concentration (mg/mL)
3.0
2.0
1.0
0.0
Total Protein
Methyltransferase
0.0
0.1
0.2
0.3
0.4
Volume 4% Protamine Sulfate/Volume Extract

Fig. 7

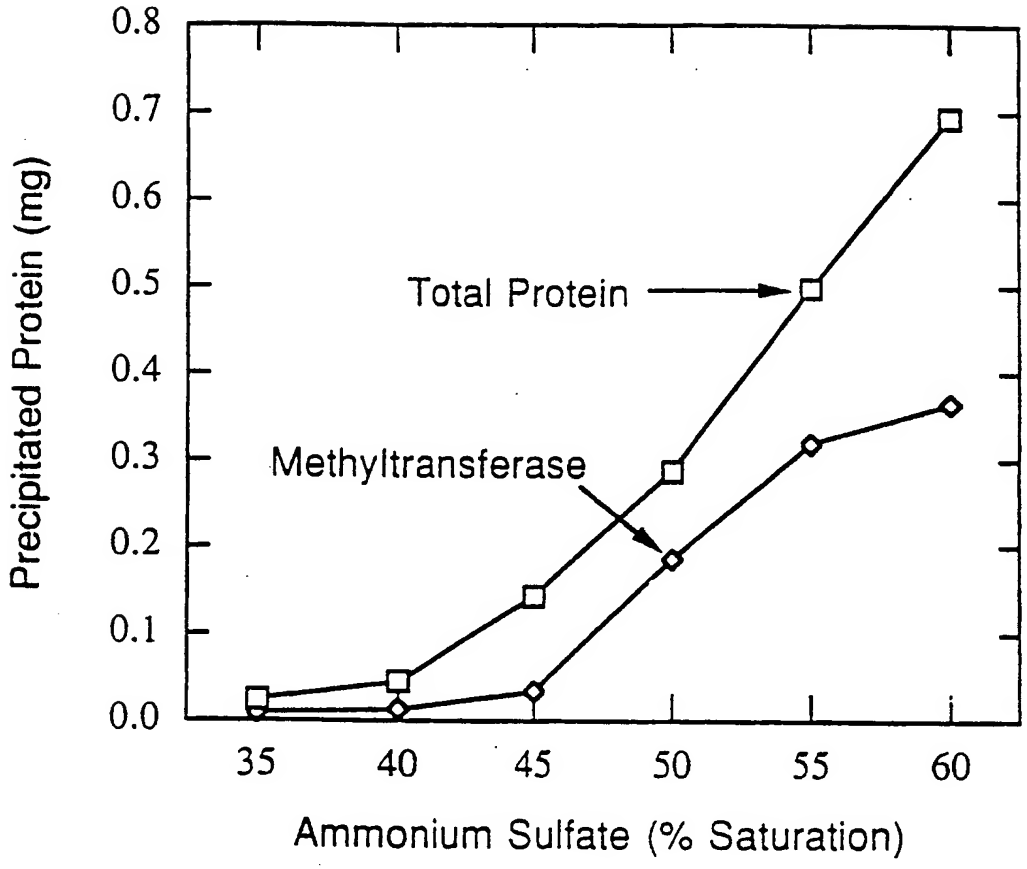

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Precipitated Protein (mg)
Total Protein
Methyltransferase
35
40
45
50
55
60
Ammonium Sulfate (% Saturation)

Fig. 8

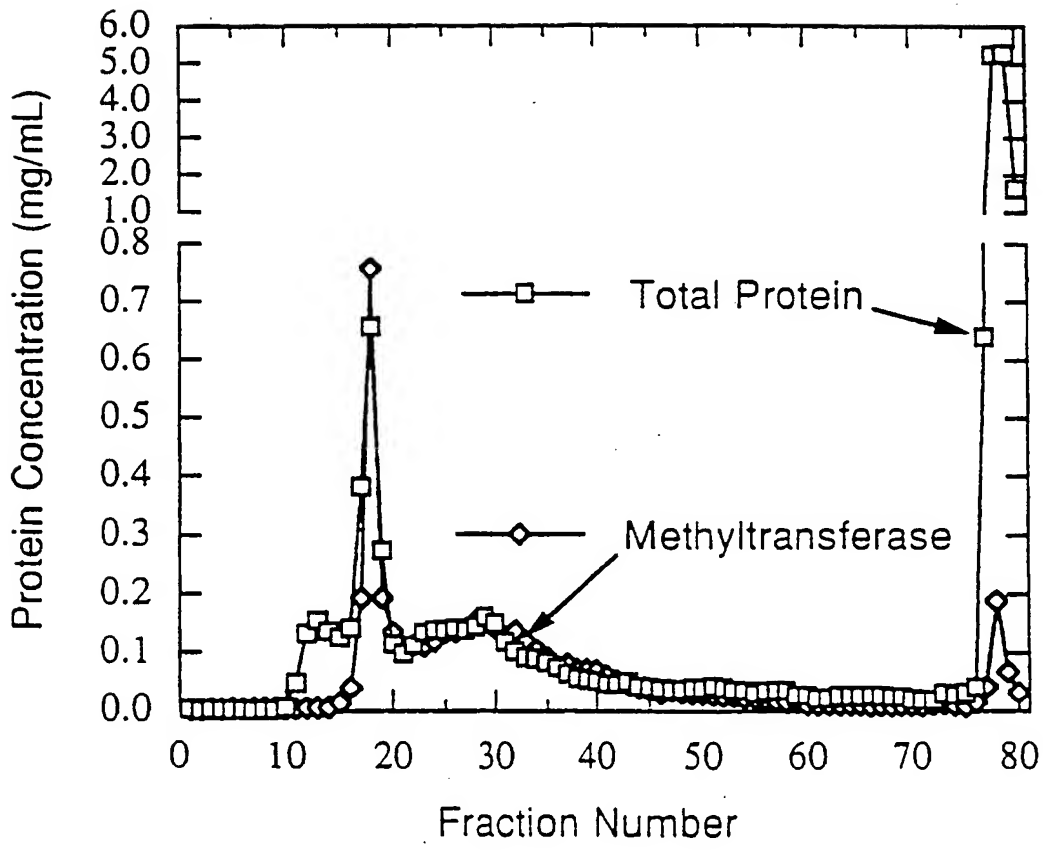

Protein Concentration (mg/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Total Protein
Methyltransferase
0
10
20
30
40
50
60
70
80
Fraction Number

Fig. 9

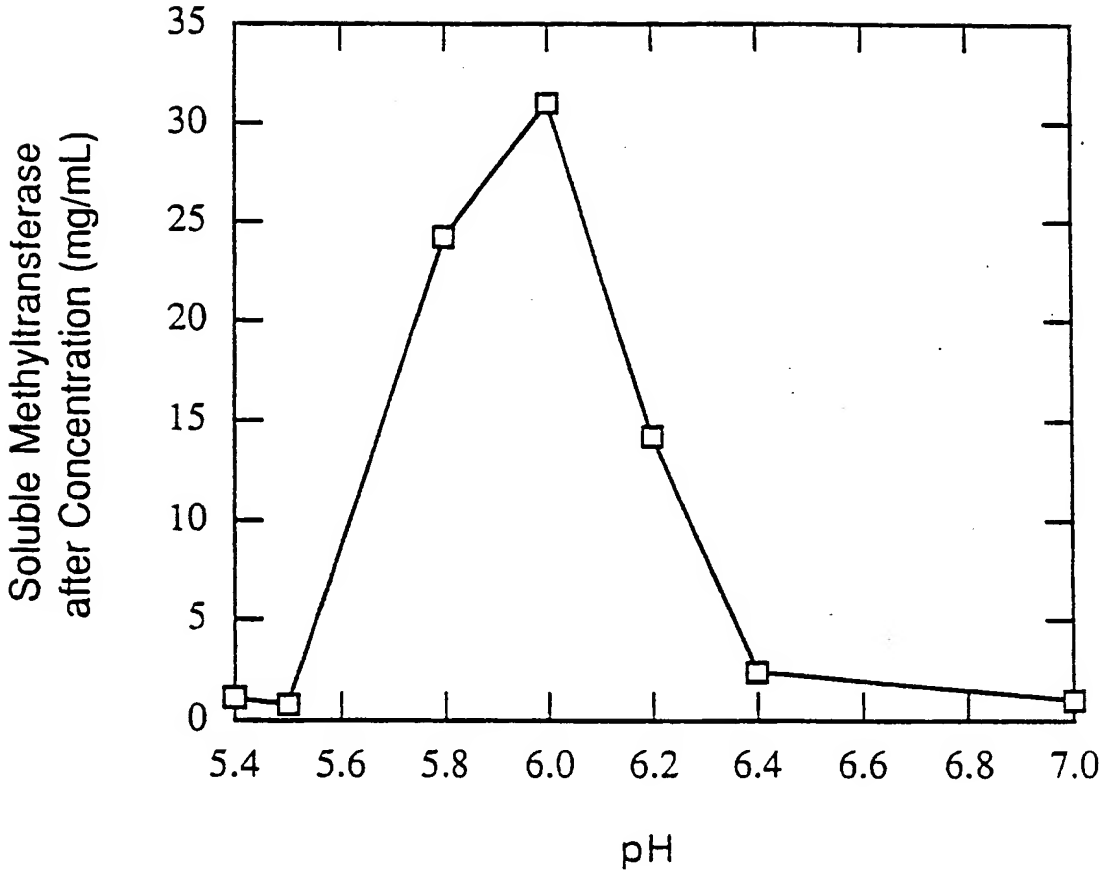

Soluble Methyltransferase after Concentration (mg/mL)
35
30
25
20
15
10
5
0
5.4
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
pH

Fig. 10

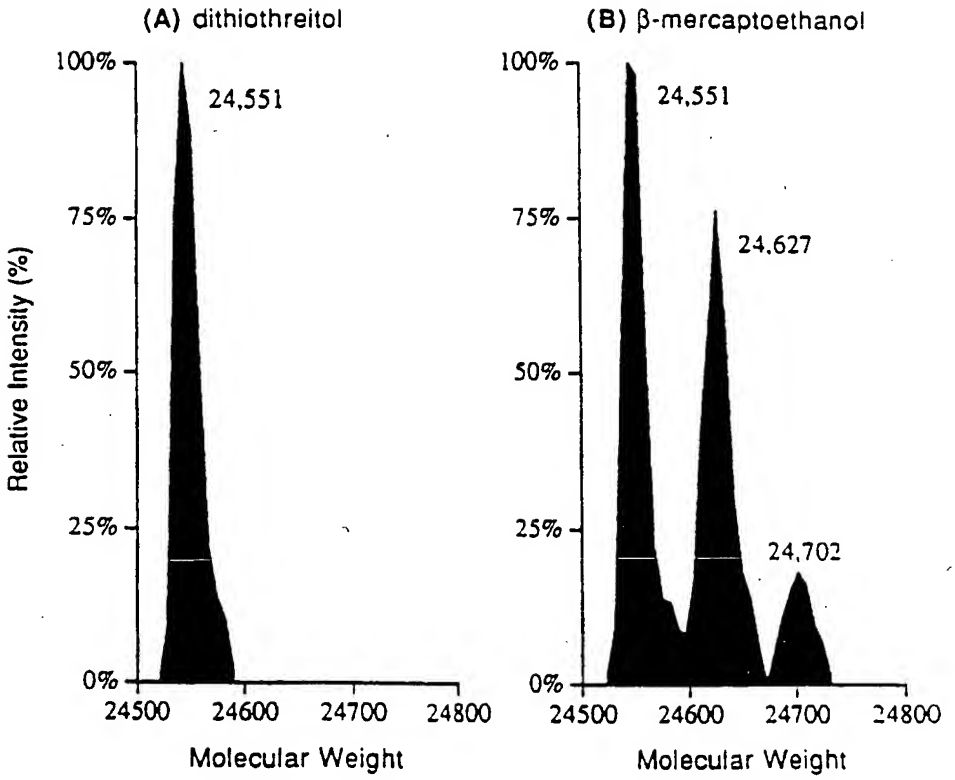

(A) dithiothreitol
(B) β-mercaptoethanol
Relative Intensity (%)
100%
75%
50%
25%
0%
24,551
24,627
24,702
24500
24600
24700
24800
Molecular Weight

Fig. 11

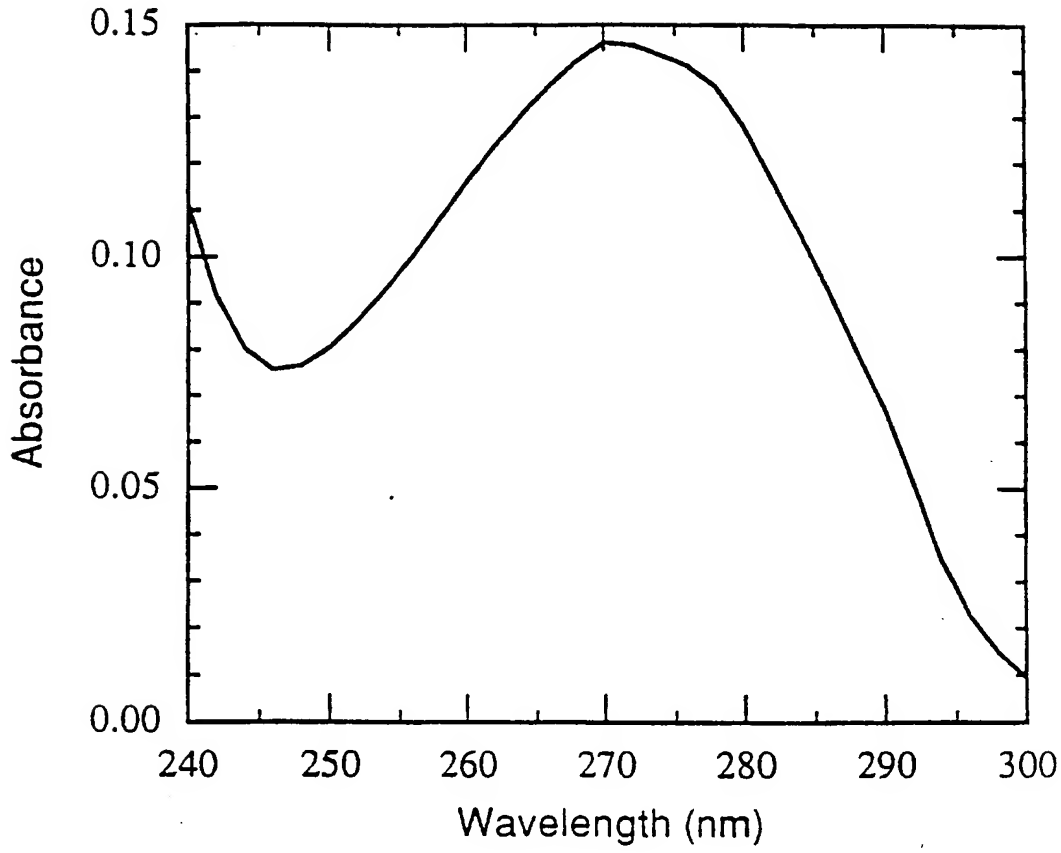

0.15
0.10
0.05
0.00
Absorbance
240
250
260
270
280
290
300
Wavelength (nm)

Fig. 12

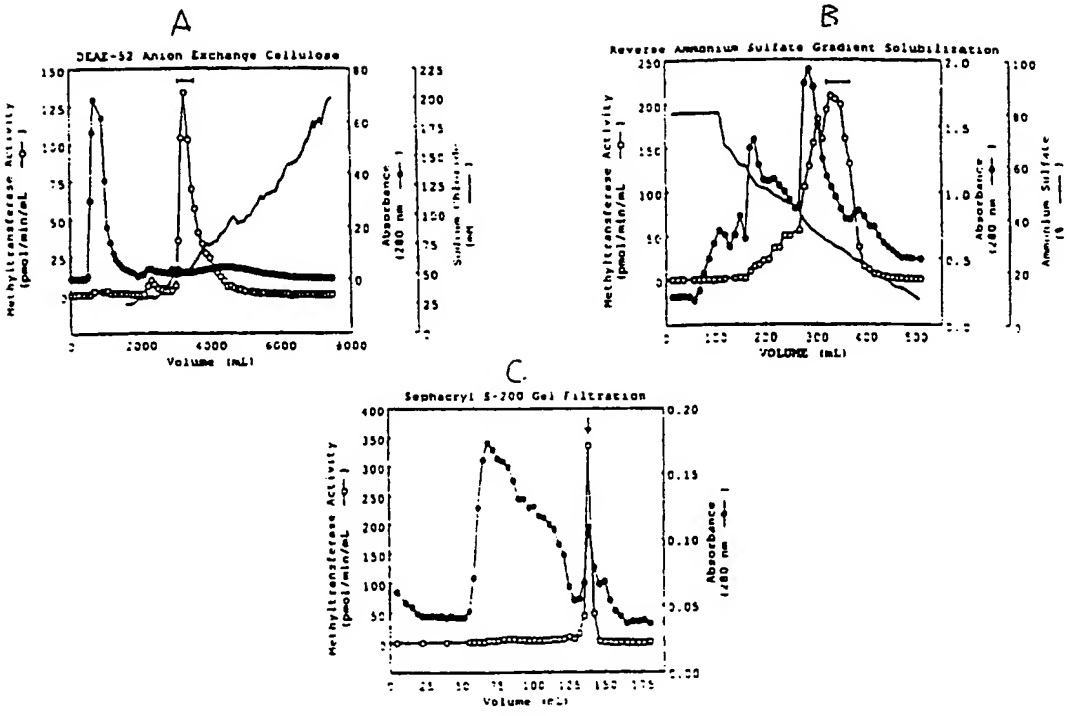

A
DEAE-52 Anion Exchange Cellulose
Methyltransferase Activity (pmol/min/mL)
Absorbance (280 nm)
Volume (mL)
B
Reverse Ammonium Sulfate Gradient Solubilization
Methyltransferase Activity (pmol/min/mL)
Absorbance (280 nm)
Ammonium Sulfate (%)
VOLUME (mL)
C.
Sephacryl S-200 Gel Filtration
Methyltransferase Activity (pmol/min/mL)
Absorbance (280 nm)
Volume (mL)

Fig.13

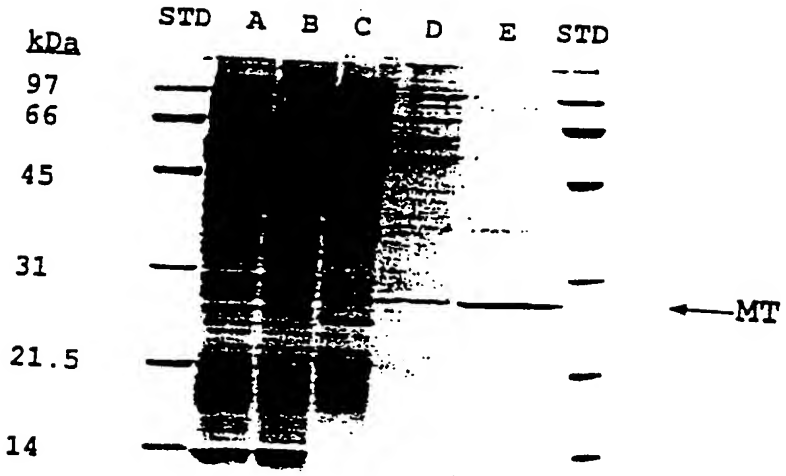


Fig.14

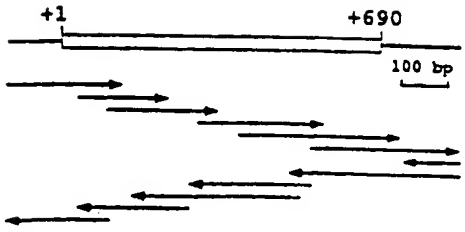
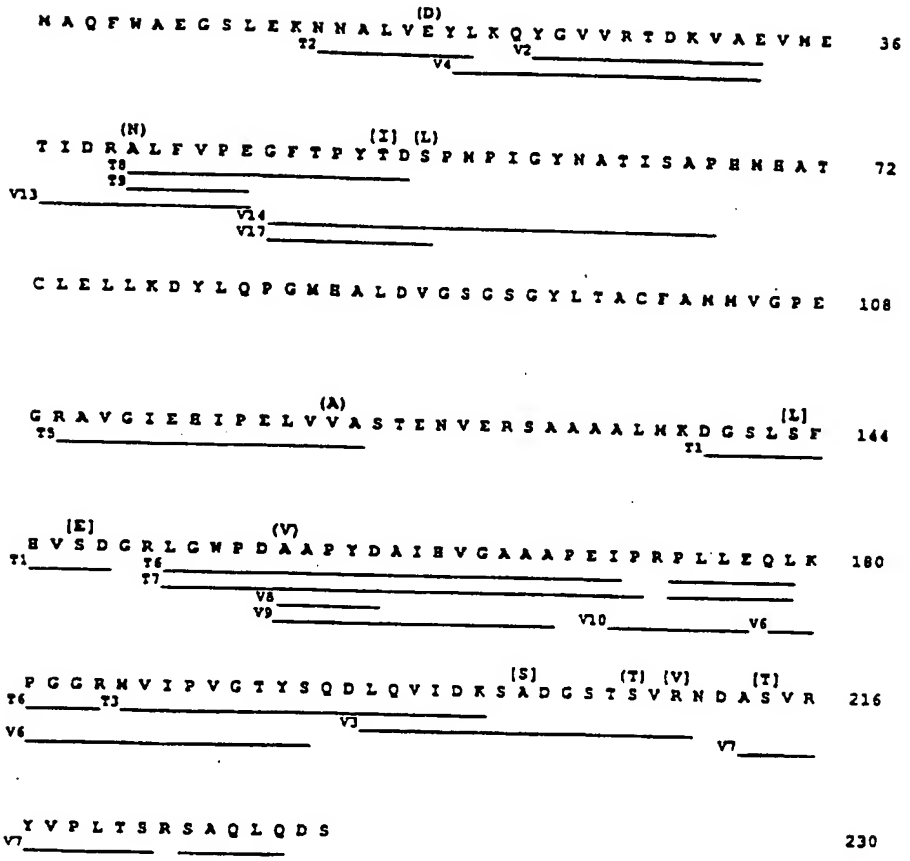


Fig.15



M A Q F W A E G S L E K N M A L V E Y L K Q Y G V V R T D K V A E V M E 36

T I D R A L F V P E G F T P Y T D S P M P I G Y N A T I S A P H M H A T 72

C L E L L K D Y L Q P G M H A L D V G S G S G Y L T A C F A M M V G P E 108

G R A V G I E H I P E L V V A S T E N V E R S A A A A L M K D G S L S F 144

H V S D G R L G W P D A A P Y D A I H V G A A A P E I P R P L L E Q L K 180

P G G R M V I P V G T Y S Q D L Q V I D K S A D G S T S V R N D A S V R 216

Y V P L T S R S A Q L Q D S 230

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13691

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 9/10, 15/63; C12P 21/06; A61K 38/51
US CL :435/69.1, 193, 320.1; 424/94.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 193, 320.1; 424/94.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; Dialog-Biosis, Embase, Life Sciences, Pascal, Medline, Toxline, WPI, Biotech Abstr., CA, SciSearch
search terms: methyltransferase, isoaspartyl, aspartyl, human

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 185, No. 1, issued 29 May 1992, MacLaren et al., "Alternative Splicing of the Human Isoaspartyl Protein Carboxyl Methyltransferase RNA Leads to the Generation of a C-terminal -RDEL Sequence in Isozyme II", pages 277-283, especially pages 280-281.	1-2 ---------- 6-19,29-44, 46-50, 58-62
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 269, No. 40, issued 07 October 1994, Brennan et al., "Repair of Spontaneously Deamidated HPr Phosphocarrier Protein Catalyzed by the L-Isoaspartate-(D-aspartate) O-Methyltransferase", pages 24586-24595, especially page 24587.	46-50, 58-62

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 FEBRUARY 1996	13 MAR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAWAI LAU Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13691

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,952,496 (STUDIER et al.) 28 August 1990, Examples 8 and 10-16.	1, 2, 6-19, 29-32, 46-50, 58-62
Y	US, A, 5,166,058 (WANG et al.) 24 November 1992, column 2, line 41 through column 3, line 2.	46-50, 58-62

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13691

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2, 6-19, 29-44, 46-50 and 58-62

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13691

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1, 2, 6-19, 29-44, 46-50 and 58-62, drawn to human methyltransferase proteins, recombinant methods for their production and purification, expression vectors encoding them, methods of using them to treat medical conditions, and pharmaceutical compositions comprising them.
Group II, claim(s) 3 and 55-57, drawn to DNA encoding plant methyltransferase proteins as well as recombinant methods of using them.
Group III, claim(s) 4, 5, 20-28, 51-54 and 63, drawn to plant methyltransferase proteins, methods for their purification, and methods of using them to treat plants.
Group IV, claim(s) 45, drawn to methods to diagnose patient status by detecting L-isoaspartyl/D-aspartyl residues.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I is drawn to the combination category of a product, methods to produce it and a method of using it. Group I has the special technical feature of nucleic acids encoding human methyltransferase, which is not shared by Groups II-IV.
Group II is drawn to the combination category of a product and a method of use. Group II has the special technical feature of nucleic acids encoding plant methyltransferase, which is not shared by Groups I and III-IV.
Group III is drawn to the combination category of a product, methods to produce it and a method of using it. Group I has the special technical feature of plant methyltransferase protein, which is not shared by Groups I-II and IV.
Group IV is drawn to the method of diagnosing patient status, which is a special technical feature not shared by Groups I-III.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.